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(57) Abstract

The present invention provides methods and compositions for screening, diagnosis and prognosis of RA, for monitoring the effectiveness of RA treatment, and for drug development. RA–Diagnostic Features (RADFs), detectable by two–dimensional electrophoresis of serum or plasma are described. The invention further provides RA–Diagnostic Protein Isoforms (RPIs) detectable in synovial fluid, serum or plasma, preparations comprising isolated RPIs, antibodies immunospecific for RPIs, and kits comprising the aforesaid.

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METHODS AND COMPOSITIONS FOR DIAGNOSIS OF RHEUMATOID ARTHRITIS

1 Introduction The present invention relates to the identification of proteins and protein isoforms that are associated with Rheumatoid Arthritis and to their use for screening, diagnosis, prognosis, therapy and drug development.

2 Background Of The Invention Rheumatoid Arthritis (RA) is a multisystem disorder in which immunological abnormalities characteristically result in symmetrical joint inflammation, articular erosions and extra-articular complications. It is the most common and disabling autoimmune arthritis, and genetic susceptibility is well defined. It affects about 1-3% of the population. Prevalence increases with age, but peaks between 30 and 55 years.

Rheumatoid factors, *i.e.*, antibodies directed against the Fc fragment of immunoglobulin G (IgG), are present in 75% of patients with RA. Rheumatoid factors may be IgM, IgG or IgA. Naturally occurring rheumatoid factors are thought to play a role in the clearance of foreign antigen from the body. In RA, rheumatoid factors are produced by synovial plasma cells and have the ability to form immune complexes, to activate complement, and to participate in the inflammatory response, suggesting that they are involved in the pathogenesis of RA. Coexistence of rheumatoid factors and reduced IG galactosylation in RA is predictive of severe disease.

Synovial tissue is the main focus of inflammation in RA and there is a chronic synovial infiltrate of CD4+ T lymphocytes, activated B-lymphocytes, mononuclear cells and

polymorphonuclear leukocytes. HLA class II expression is increased and is found on nearly all cell types, indicating that they are in an active state. Chronic disease is characterized by erosion of cartilage and bone, and synovial hypertrophy.

Diagnosis of RA has been difficult. A disease pattern may not be evident from the history, examination and investigations in early disease. There is no diagnostic test for RA, but the American College of Rheumatology has defined criteria for its classification (*Medicine*, ed. Axford, J., Blackwell Science, 1996, pp. 3.18-3.22). These criteria are merely observational landmarks which, in combination, can be deemed to indicate RA.

75% of adults with RA are IgM rheumatoid factor positive. This may be absent early in the disease. The level of rheumatoid factor may be used prognostically at diagnosis, but fluctuations are unhelpful in monitoring the disease. Antinuclear antibodies are present in 30% of patients.

IgM rheumatoid factor is usually measured using agglutination tests. These include the latex test in which particles coated with IgG are agglutinated, and the sheep-cell agglutination test (SCAT or Rose-Waaler test), in which sheep red cells are agglutinated. At least 70% of RA patients diagnosed by other criteria have a positive rheumatoid factor latex agglutination test. Rheumatoid factor tests are also positive in other rheumatic diseases, viral infections, chronic inflammatory diseases, neoplasm or chemotherapy and, significantly, 4% of healthy individuals.

Drug treatment of RA is aimed at: (1) alleviating pain (analgesics); (2) modifying the inflammatory events themselves once they have been triggered (anti-inflammatory

drugs.; and (3) modifying the immunological events leading to inflammation (disease-modifying drugs).

3 Summary Of The Invention The present invention provides methods and compositions for screening, diagnosis and prognosis of RA, for monitoring the effectiveness of RA treatment, for therapy of RA and for drug development.

4

A first aspect of the invention provides methods for diagnosis of RA that comprise analyzing a sample (e.g., plasma, serum, or synovial fluid) by two-dimensional electrophoresis to detect the level of at least one Rheumatoid Arthritis-Diagnostic Feature (RADF), e.g. an RADF selected from the group of RADFs disclosed herein. These methods are also suitable for screening, prognosis, monitoring the results of therapy, and drug development.

A second aspect of the invention provides methods for diagnosis of RA that comprise detecting in a sample (e.g., plasma, serum, or synovial fluid) the level of at least one Rheumatoid Arthritis-Diagnostic Protein Isoform (RPI), e.g. an RPI selected from the group of RPIs disclosed herein. These methods are also suitable for screening, prognosis, monitoring the results of therapy, and drug development.

A third aspect of the invention provides monoclonal and polyclonal antibodies capable of immunospecific binding to an RPI, e.g. an RPI disclosed herein.

A fourth aspect of the invention provides a preparation comprising an isolated RPI, *i.e.*, an RPI free from proteins or protein isoforms having a significantly different isoelectric point or a significantly different apparent molecular weight from the RPI.

A fifth aspect of the invention provides methods for treatment or prevention of RA that comprise administering a

compound which is able to decrease the level of at least one RADF -- or the level or activity of at least one RPI -- that is present at an increased level in the synovial fluid of RA patients as compared with the serum of RA patients. Preferably, the administered compound is an antibody, an anti-sense oligonucleotide, a ribozyme, or an oligonucleotide capable of forming a triple helix.

A sixth aspect of the invention provides methods for treatment or prevention of RA that comprise administering a compound which is able to increase the level of at least one RADF -- or the level or activity of at least one RPI -- that is present at a decreased level in the synovial fluid of RA patients as compared with the serum of RA patients. Preferably, the administered compound is an RPI, a nucleic acid encoding an RPI, (e.g. a nucleic acid that is part of an expression vector), or a cell that is able to express and secrete one or more RPIs.

Brief Description Of The Figures Figure 1 is an image obtained from 2-dimensional electrophoresis of normal human serum, which has been annotated to identify 14 landmark features, designated PL1 to PL16.

Detailed Description Of The Invention The invention described in detail below encompasses methods and compositions for screening, diagnosis and prognosis of RA in a subject, methods for monitoring the results of RA therapy, methods for drug development, and methods for treating RA. Preferably, the subject is a mammal, more preferably a human, and most preferably a human adult.

For clarity of disclosure, and not by way of limitation, the invention will be described with respect to the analysis of serum and synovial fluid samples. However, as one skilled

in the art will appreciate, the assays and techniques described herein can be applied to other types of patient samples, including a body fluid (e.g., plasma, urine, cerebrospinal fluid, joint aspirate), a tissue sample, or homogenate thereof.

5.1 Rheumatoid Arthritis-Diagnostic Features (RADFs)

In one aspect of the invention, two-dimensional electrophoresis is used to analyze serum from a subject in order to measure the abundance of one or more Rheumatoid Arthritis-Diagnostic Features (RADFs) for screening or diagnosis of RA, to determine the prognosis of an RA patient, to monitor the effectiveness of RA therapy or for drug development. As used herein, "two-dimensional electrophoresis" (2D-electrophoresis) means a technique comprising isoelectric focusing, followed by denaturing electrophoresis; this generates a two-dimensional gel (2D-gel) containing a plurality of separated proteins. Preferably, the step of denaturing electrophoresis uses polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Especially preferred are the highly accurate and automatable methods and apparatus ("the Preferred Technology") described in U.S. Application No. 08/980,574, which is incorporated herein by reference in its entirety. Briefly, the Preferred Technology provides efficient, computer-assisted methods and apparatus for identifying, selecting and characterizing biomolecules in a biological sample. A two-dimensional array is generated by separating biomolecules on a two-

dimensional gel according to their electrophoretic mobility and isoelectric point. A computer-generated digital profile of the array is generated, representing the identity, apparent molecular weight, isoelectric point, and relative abundance of a plurality of biomolecules detected in the two-dimensional array, thereby permitting computer-mediated comparison of profiles from multiple biological samples, as well as computer aided excision of separated proteins of interest.

As used herein, the term "Rheumatoid Arthritis-Diagnostic Feature" (RADF) refers to a feature (*e.g.*, a spot in a 2D gel), detectable by 2D electrophoresis of a biological sample, that is differentially present in one sample compared to another, relevant sample, *e.g.*, (1) in serum from a subject with RA compared with serum from a subject without RA or (2) in synovial fluid taken from a subject with RA compared with serum taken from a subject with RA.

As used herein, a feature (or a protein isoform) is "differentially present" in a first sample with respect to a second sample when a method for detecting the feature or isoform (*e.g.*, 2D electrophoresis or an immunoassay) reveals that the feature (or protein isoform) is present at a different relative abundance in a first sample as compared with a second sample. If the measured feature in the first sample is at a higher relative abundance than in the second sample, the feature or isoform is "increased" in the first sample with respect to the second; conversely, if the measured feature in the first sample is at a lower relative abundance than in the second sample, the feature or isoform

is "decreased" in the first sample with respect to the second.

Preferably, the relative abundance of a feature in two samples is determined in two steps. First, the signal obtained upon detecting the feature in a sample is normalized by reference to a suitable background parameter, e.g., to the total protein in the sample being analyzed (e.g., total protein loaded onto a gel), to an invariant feature, i.e., a feature whose abundance is known to be similar in the samples being compared, e.g., one or more Expression Reference Features (ERFs), such as the ERFs disclosed below, or to the total signal detected from all proteins in the sample.

Secondly, the normalized signal for the feature in one sample or sample set is compared with the normalized signal for the same feature in another sample or sample set in order to identify features that are "differentially present" in the first sample (or sample set) with respect to the second.

The RADFs disclosed herein have been identified by one of two sample comparisons. The first group of RADFs are those features that (a) are differentially present in the synovial fluid of subjects with RA as compared with the serum of subjects with RA, and (b) are not differentially present in the synovial fluid of subjects without RA as compared with the serum of subjects without RA. Subjects without RA can include normal subjects with no known disease or condition, or subjects with joint diseases or conditions other than RA, including gout, osteoarthritis, or synovitis (e.g., traumatic synovitis). The second group of RADFs are those features that are differentially present in serum from a subject with RA compared with serum from a subject without RA.

Four groups of RADFs have been identified through the methods and apparatus of the Preferred Technology. The first

group consists of RADFs that are increased in synovial fluid verses serum in subjects with RA, but are not increased in synovial fluid versus serum in subjects without RA. These RADFs can be described by apparent molecular weight (MW) and isolectric point (pI), as follows:

Table I. RADFs Increased in RA Synovial Fluid vs. RA Serum

Name	Fold increase	pI	MW (kd)
RADF-1	118.3	6.76	74,447
RADF-2	94.7	7.42	25,049
RADF-3	62.5	4.92	54,948
RADF-4	26.0	5.1	53,241
RADF-5	20.1	6.94	27,221
RADF-6	11.0	5.14	137,225
RADF-7	9.9	5.86	26,217
RADF-8	8.2	5.79	58,161
RADF-9	5.0	6.17	57,613
RADF-10	3.7	5.43	39,842
RADF-11	3.4	5.98	52,631
RADF-12	3.3	7.06	72,543

The second group consists of RADFs that are decreased in synovial fluid of versus serum in subjects with RA, but are not decreased the the synovial fluid versus serum in subjects without RA. These RADFs can be described by apparent molecular weight (MW) and isolectric point (pI), as follows:

Table II. RADFs Decreased in RA Synovial Fluid vs. RA Serum

Name	Fold decrease	pI	MW (kd)
RADF-13	37.5	4.92	53,578

RADF-14	14.6	6.2	76,783
RADF-15	12.6	4.56	63,737
RADF-16	11.9	5.36	24,124
RADF-17	10.1	5.96	158,868
RADF-18	9.0	9.52	14,953
RADF-19	5.3	5.1	131,608
RADF-20	3.7	5.12	60,216
RADF-21	2.9	4.95	32,321
RADF-22	3.0	6.95	27,812

The third group consists of RADFs that are increased in the serum of subjects with RA as compared with the serum of subjects without RA. These RADFs can be described by apparent molecular weight (MW) and isoelectric point (pI) as follows:

Table III. RADFs Increased In RA Serum vs. Non-RA Serum

Name	Fold increase	pI	MW (kd)
RADF-13	2.7	4.92	53,578
RADF-16	4.3	5.36	24,124
RADF-22	2.7	6.95	27,812
RADF-23	10.1	9.00	47,978
RADF-24	5.5	5.31	74,447
RADF-25	3.5	5.34	40,271
RADF-26	3.4	4.81	40,997
RADF-27	3.2	6.97	56,354
RADF-28	3.1	5.41	16,807
RADF-29	2.9	5.02	36,372
RADF-30	2.8	4.52	17,629

RADF-31	2.6	5.26	16,687

The fourth group consists of RADFs that are decreased in the serum of subjects with RA as compared with the serum of subjects without RA. These RADFs can be described by apparent molecular weight (MW) and isoelectric point (pI) as follows:

Table IV. RADFs Decreased In RA Serum vs. Non-RA Serum

Name	Fold decrease	pI	MW (kd)
RADF-32	36.5	6.61	70,511
RADF-33	4.3	6.29	76,112
RADF-34	3.7	5.65	37,966
RADF-35	3.3	5.93	34,471
RADF-36	3.1	6.09	57,613
RADF-37	3.0	5.41	183,864
RADF-38	2.9	5.04	81,696
RADF-39	2.8	6.25	53,917
RADF-40	2.6	6.37	82,423

For any given RADF in the first and second groups the ratio obtained upon comparing the signal measured in synovial fluid from subjects with RA relative to the signal measured in serum from subjects with RA will depend upon the particular analytical protocol and detection technique that is used. Accordingly, the present invention contemplates that for each RADF in the first and second groups, a laboratory will establish reference ranges for the ratio of each RADF in synovial fluid versus serum in subjects with and without RA, according to the analytical protocol and detection technique in use, as is conventional in the diagnostic art. Preferably, at least one control pair of synovial fluid and serum samples from a subject known to have RA, or at least one control pair of synovial fluid and serum samples from a subject known not to have RA (and more preferably at least one of each such control pairs) is included with each batch of test samples analyzed.

Similarly, for any given RADF in the third and fourth groups, the ratio obtained upon comparing the signal measured in serum from subjects with RA relative to the signal measured in serum from subjects without RA will depend upon the particular analytical protocol and detection technique that is used. Accordingly, the present invention contemplates that each laboratory will establish reference ranges for the ratio of each RADF in the third and fourth groups in subjects with and without RA according to the analytical protocol and detection technique in use, as is conventional in the diagnostic art. Preferably, at least one positive control serum sample from a subject known to have RA, or a negative control serum sample from a subject known to be free of RA (and more preferably at least one positive

and at least one negative control sample) is included with each batch of test samples analyzed.

In a preferred embodiment, the signal associated with an RADF is normalized with reference to one or more Expression Reference Features (ERFs) detected in the same 2D gel. As will be apparent to one of ordinary skill in the art, such ERFs may readily be determined by comparing different samples using the Preferred Technology. Suitable ERFs for this purpose include (but are not limited to) those described in the following table:

TABLE V

ERF-#	MW	pI
ERF-1	38719	5.34
ERF-2	13915	5.26
ERF-3	73465	5.14
ERF-4	40924	4.87
ERF-5	104893	7.22
ERF-6	37294	7.62
ERF-7	24124	5.48
ERF-8	53409	4.56
ERF-9	36372	5.02
ERF-10	26930	5.52

By way of example, Table VI shows the levels of the RADFs identified in Tables I and II normalized to ERF-1 and ERF-3; one of skill in the art will realize that the levels of RADFs can be normalized relative to any desired ERF. The values shown in Table VI are the ratios of each RADF relative to the ERF in question, where a positive ratio indicates enhanced levels of the RADF relative to the ERF, and a

negative ratio indicates diminished levels of the RADF relative to the ERF. As will be evident to one of skill in the art, the RADF/ERF ratios can serve as a diagnostic parameters in their own right.

TABLE VI Ratio of RADFs to ERFs in RA Synovial Fluid

RADF#	Normalized to ERF-1	Normalized to ERF-3
RADF-1	-22.2	-26.0
RADF-2	-96.6	-124.6
RADF-3	-59.4	-71.3
RADF-13	38.1	31.8
RADF-4	-23.0	-27.6
RADF-14	25.2	21.9
RADF-5	-6.4	-7.3
RADF-15	13.2	11.1
RADF-16	12.3	9.9
RADF-6	-10.3	-12.2
RADF-17	10.2	8.4
RADF-7	-2.3	-2.8
RADF-18	9.2	7.6
RADF-8	-7.3	-9.2
RADF-19	5.5	4.6
RADF-9	-4.8	-5.6
RADF-20	3.8	3.3
RADF-10	-3.7	-4.4
RADF-11	-3.5	-4.7
RADF-12	-2.5	-3.1
RADF-22	2.7	2.3

In similar fashion Table VII shows the levels of RADFs identified in Tables III and IV normalized to ERF-1 and ERF-3. One of skill in the art will realize that the levels of RADFs can be expressed relative to any desired ERF, and that the RADF/ERF ratios can be used as diagnostic parameters in their own right.

TABLE VII Ratio of RADFs to ERFs in RA Serum

RADF#	Normalized to ERF-1	Normalized to ERF-3
RADF-32	-13.4	-9.5
RADF-23	5.6	7.3
RADF-24	4.4	5.3
RADF-16	3.5	4.0
RADF-33	-1.4	-1.3
RADF-34	-2.9	-2.5
RADF-25	2.9	3.2
RADF-26	2.6	2.8
RADF-35	-4.0	-3.7
RADF-27	2.4	2.6
RADF-36	-3.0	-2.6
RADF-28	-2.2	2.8
RADF-37	-4.2	-3.5

As the skilled artisan will appreciate, the measured MW and pI of a given feature or protein isoform will vary to some extent depending on the precise protocol used for each step of the 2D electrophoresis and for landmark matching. As used herein, the terms "MW" and "pI" are defined, respectively, to mean the apparent molecular weight and the isoelectric point of a feature or protein isoform as measured

in exact accordance with the experimental protocol set forth in Section 6 below ("the Reference Protocol"). When the Reference Protocol is followed and when samples are run in duplicate or a higher number of replicates, variation in the measured mean pI of an RADF or RPI is typically less than ±1% and variation in the measured mean MW of an RADF or RPI is typically less than ±5%. Where the skilled artisan wishes to deviate from the Reference Protocol, calibration experiments should be performed to compare the MW and pI for each RADF or protein isoform as detected (a) by the Reference Protocol and (b) by the deviant protocol.

RADFs can be used for detection, prognosis, diagnosis, or monitoring of RA or for drug development. In one embodiment of the invention, synovial fluid and serum from a subject are analyzed by 2D electrophoresis for quantitative detection of one or more RADFs selected from the group consisting of RADF-1 to RADF-12, wherein an increased abundance of an RADF in synovial fluid relative to serum indicates the presence of RA.

In another embodiment of the invention, synovial fluid and serum from a subject are analyzed by 2D electrophoresis for quantitative detection of one or more RADFs selected from the group consisting of RADF-13 to RADF-22 wherein a decreased abundance of an RADF in synovial fluid relative to serum indicates the presence of RA.

In another embodiment of the invention, synovial fluid from a subject is analyzed by 2D electrophoresis for quantitative detection of one or more RADFs selected from the group consisting of RADF-1 to RADF-22 wherein the ratio of the one or more RADFs relative to an Expression Reference Feature (ERF) indicates the presence of RA; ERF-1 and ERF-3 are preferred ERFs for this purpose.

Table VIII. RPI's Increased In Synovial Fluid

RPI	RADF	Homologous Protein	Partial Amino Acid Sequences	pI	MW (kd)
RPI-1	RADF-1	Transferrin	DQYELLCLDNTR EGYYGYTGAFR DYELLCLDGTR CQSFR INHCR SCHTGLGR SCHTAVGR APNHAVVTR ASYLDCIR WCALSHHER DSGFQMNQLR SASDLTWDNLK WCAVSEHEATK KDSGFQMNQLR MYLGYEYVTAIR MYLGYEYVTAIR CSTSSLLEACTFR FDEFFSEGCAPGSK KPVEEYANCHLAR EDPQTFYYAVAVVK DCHLAQVPSHTVVAR ELDIWELLNQAQEHFGK SAGWNIPIGLLYCDLPEPR	6.76	74,447
RPI-2	RADF-2	IgG κ light chain	SGTASVVCLLNNFYPR FSGSGSGTDFTLTISR EIVLTQSPATLSLSPGER	7.42	25,049
RPI-3	RADF-3	Alpha-1-antitrypsin	WERPFEVK SVLGQLGITK LSITGTYDLK SPLFMGK FLENEDR QINDYVEK KQINDYVEK LGMFNIQHCK GKWERPFEVK TDTSHHDQDHPTFNK LQHLENELTHDIITK VFSNGADLSGVTEEAPLK LYHSEAFTVNFGDTEEAK	4.92	54,948
RPI-4	RADF-4	Vitamin D binding protein	YTFELSR FEDCCQEK HLSLLTTLSNR VCSQYAAYGEK RTHLPEVFLSK	5.1	53,241
RPI-5	RADF-6	Ceruloplasmin precursor	EYTDASFTNR DDEEFIESNK QSEDSTFYLGER ALYLQYTDETFR LISVDTEHSNIYLQNGPDR QYTDSTFR MYYSAVDPTK GAYPLSIEPIGVR NNEGTYYSPNYNPQSR	5.14	137,225
RPI-6	RADF-8	Ig alpha-1&2 chain c region	WLQGSQELPR YLTWASR QEPSQGTTTFAVTSILR	5.79	58,161
RPI-7	RADF-9	Ig alpha-1&2 chain	WLQGSQELPR	6.17	57,613

		C region			
RPI-8	RADF-10	Haptoglobin precurso	VGYVSGWGR YVMLPVADQDQCIR	5.43	39,642
RPI-9	RADF-11	Beta-2-glycoprotein precursor (Apo-lipoprotein H)	VCPFAGILENGAVR ATVYQGER EHSSLAFWK TCPFDEDLPFSTVVPLK	5.98	50,631
RPI-10	RADF-11	NOVEL	VAAGIVLVEHFGR	5.98	50,631
RPI-11	RADF-12	Transferrin	DYELLCLDGTR	7.06	72,543

The second group consists of RPIs that are decreased in synovial fluid verses serum serum in subjects with RA, but are not decreased in synovial fluid verses serum in subjects without RA. The MWs, pIs and partial amino acid sequences of these RPIs are presented in Table IX, as follows:

Table IX.

RPI	RADF	Homologous Protein	Partial Amino Acid Sequences	pI	MW (kd)
RPI-12	RADF-13	Alpha-1-antitrypsin	ITEEEDFHVDQVTTVK VFSNGADLSGVTEEAPLK LSITGTYDLK LGMFNIQHCK KVLGQLGITK FLENEDR FLENEDRR MERPFEVK FKWERPFEVK RLYHSEAFTVNFGDTEEAK LYHSEAFTVNFGDTEEAKK	4.92	53,578
RPI-13	RADF-14	Transferrin precursor	LSGFQMNQLR WCALSHHER CASDLTWDNLK LGYYGYTGAFR LCHTGLGR SCHTAVGR APNHAVVTR SCHLAQVPSHTVVAR	6.2	76,789
RPI-14	RADF-14	IgM chain	LICQATGFSPR VSVFVPPR IGFFGNPR LIQVSWLR LVGSGVTTDQVQAEAK FTCTVTHTDLPSPLK TVALHRPDVYLLPPAR IQLNLR GFPSVLR VQHPNGNK VNPLPVIAELPPK VNMQGTDEHVVCK VVTSAPMPEPQAPGR	6.2	76,789

RPI	RADF	Homologous Protein	Partial Amino Acid Sequences	pI	MW (kd)
			ESDWLSQSMFTCR		
RPI-15	RADF-15	Alpha-1-antichymotrypsin precursor	DSLEFR ADLSGITGAR EQLSLLDR ITLLSALVETR WEMPFDPQDTHQSR LYGSEAFATDFQDSAAAK AVLDVFEEGTEASAATAVK	4.56	63,737
RPI-16	RADF-16	Apolipoprotein A-1 precursor	LSPLGEEMR VQPYLDDFQK DLATVYVDVLK AHVDALR AELQEGAR WQEEMELYR VSFLSALEEYTK	5.36	24,124
RPI-17	RADF-16	NOVEL	DSGAD(I/L)S	5.36	24,124
RPI-18	RADF-17	Complement factor H precursor	QMSKYPSGER MDGASNVTCINSR CGKDGWSAQPTCIK GNTAKCTSTGWIPAPR IDVHLVPDR EFDHNSNIR RPYFPVAVGK SLGNVIMVCR LYSTCEGGFR HGGLYHENMR EIMENYNIALR TDCLSLPSFENAIPMGEK	5.96	158,868
RPI-19	RADF-17	Copper transporting ATPase	DRSASHLDHK ASINSLLSDKR QIEAMGFPAFVK VFAEVLPSHKVAK CYIQVTGMTCASCVANIER	5.96	158,868
RPI-20	RADF-17	NOVEL	NV(I/L)DAPHAR	5.96	158,868
RPI-21	RADF-18	Hemoglobin alpha chain	VGAHAGEYGAEALER MFLSFPTTK TYFPHFDLSHGSAQVK	9.52	14,953
RPI-22	RADF-19	Ceruloplasmin precursor	GAYPLSIEPIGVR ALYLQYTDETFR GSLHANGR YTVNQCR QYTDSTFR DNEDFQESNR QSEDSTFYLGER DLYSGLIGPLIVCR VDKDNEDFQESNR NNEGTYYSPNYNPQSR	5.1	131,608
RPI-23	RADF-20	Ig alpha-1&2 chain c region	QEPSQGTTTFAVTSILR YLTWASR SAVQGPPER WLQGSQELPR DASGATFTWTPSSGK	5.12	60,216
RPI-24	RADF-22	Complement C3C precursor	HQQTVTIPPK SSLSVPYVIVPLK LPYSVVR DSITTWEILAVSMSDK SEFPESWLWNVEDLK TLDPER VVPEGIR NEQVEIR	6.95	27,812

RPI	RADF	Homologous Protein	Partial Amino Acid Sequences	pI	MW (kd
			AVLYNYR RHQQTVTIPPK AAVYHHFISDGVF VELLHNPAFCSLATTF SNLDEDIIAEENIVSF		

The third group comprises RPIs that are increased in the serum of subjects with RA as compared with the serum of subjects without RA. The MWs, pIs and partial amino acid sequences of these RPIs are presented in Table X, as follows:

Table X.

RPI	RADF	Homologous Protein	Partial Amino Acid Sequence	pI	MW (kd)
RPI-25	RADF-23	Ig γ chain C region	EPQVYTLPPSR DTLMISR GFSVFPLAPSSK STSGGTAALGCLVK FNWYVDGVEVHNAK TPEVTCVVVDVSHEDPEVF	9	47,978
RPI-26	RADF-24	Hemopexin	NFPSPVDAAFR GGYTLVSGYPK GECQAEGVLFFQGDR	5.31	74,447
RPI-16	RADF-16	Apolipoprotein A-1 precursor	LSPLGEEMR VQPYLDDFQK DLATVYVDVLK AHVDALR AELQEGAR WQEEMELYR VSFLSALEEYTK	5.36	24,124
RPI-17	RADF-16	NOVEL	DSGAD(I/L)S	5.36	24,124
RPI-27	RADF-25	Haptoglobin precursor	VGYVSGWGR GSFPWQAK YVMLPVADQDQCIR VTSIQDWVQK SCAVAEYGVYVK	5.34	40,271
RPI-28	RADF-26	Complement C3 precursor	ENEGFTVTAEGK VYAYYNLEESCTR NTMILEICTR VSHSEDDCLAFK	4.81	40,997
RPI-29	RADF-26	Haptoglobin precursor	VGYVSGWGR	4.81	40,997
RPI-30	RADF-26	Zn alpha2 glyco-protein	QDSQLQK IDVHWTR SQPMGLWR WEAEPVYVQR AREDIFMETLK AYLEEECPATLR	4.81	40,997
RPI-31	RADF-27	Ig alpha-1&2	WLQGSQELPR	6.97	56,354

RPI	RADF	Homologous Protein	Partial Amino Acid Sequence	pI	MW (kd)
		chain c receptor			
RPI-32	RADF-27	Complement factor B (BB Fragment)	QVFAHAR ISVIRPSK VASYGVKPR DISEVVTPR GDSGGPLIVHK YGLVTYATYPK LFFTTTCQQQK FLCTGGVSPYADPNTCR	6.97	56,354
RPI-12	RADF-13	Alpha-1-antitrypsin	DTEEEDFHVDQVTTVK VFSNGADLSGVTEEAPLK LSITGTYDLK LGMFNIQHCK SVLGQLGITK FLENEDR FLENEDRR WERPFEVK GKWERPFEVK KLYHSEAFTVNFGDTEEAK	4.92	53,578
RPI-24	RADF-22	Complement C3C precursor	HQQTVTIPPK SSLSVPYVIVPLK LFYSVVR DSITTWEILAVSMSDK SEFPESWLWNVEDLK TLDPER VVFEGIR NEQVEIR AVLYNYR RHQQTVTIPPK AAVYHHFISDGVR VELLHNPAFCSLATTK SNLDEDIIAEENIVSR	6.95	27,812

The fourth group comprises RPIs that are decreased in the serum of subjects with RA as compared with the serum of subjects without RA. The MWs, pIs and partial amino acid sequences of these RPIs are presented in Table XI, as follows:

Table XI.

RPI	RADF	Homologous Protein	Partial Amino Acid Sequence	pI	MW (kd)
RPI-33	RADF-32	Transferrin	DSGFQMNQLR	6.61	70,511
RPI-34	RADF-33	Transferrin	DSGFQMNQLR CDEWSVNSVGK	6.29	76,112
RPI-35	RADF-34	C-terminal tryptic fragment Complement factor H	CTSTGWIPAPR SCDNPYIPNGDYSPLR EYHFGQAVR SLGNVIMVCR TGDEITYQCR	5.65	37,966

RPI	RADF	Homologous Protein	Partial Amino Acid Sequence	pI	MW (kd
			PGEWVALNPLR GEWVALNPLR		
RPI-36	RADF-35	Haptoglobin related protein precursor	VGYVSGWGQSDNFK NYAEVGR VVLHPNYHQVDIGLIK	5.93	34,471
RPI-37	RADF-36	Ig alpha-1&2 chain c region	WLQGSQELPR QEPSQGTTTFAVTSILR YLTWASR SAVQGPPER	6.09	57,613
RPI-38	RADF-37	Complement factor H precursor	EIMENYNIALR IDVHLVPDR EFDHNSNIR RPYFPVAVGK SLGNVIMVCR SCDIPVFMNAR TDCLSLPSFENAIPMGEK	5.41	183,664

In one embodiment of the invention, synovial fluid and serum from a subject are analyzed for quantitative detection of one or more RPIs selected from the group consisting of RPI-1 to RPI-11 wherein an increased abundance of one or more such RPIs in synovial fluid relative to serum indicates the presence of RA. In a further embodiment of the invention, synovial fluid and serum from a subject are analyzed for quantitative detection of one or more RPIs selected from the group consisting of RPI-12 to RPI-24 wherein a decreased abundance of one or more such RPIs in synovial fluid relative to serum indicates the presence of RA.

In another embodiment of the invention, serum from a subject is analyzed for quantitative detection of one or more RPIs selected from the group consisting of RPI-12, RPI-16, RPI-17 and RPI-24 to RPI-32, wherein an increased abundance

of one or more such RPIs in serum from the subject indicates the presence of RA. In another embodiment of the invention, serum from a subject is analyzed for quantitative detection of one or more RPIs selected from the group consisting of RPI-33 to RPI-38, wherein a decreased abundance of one or more such RPIs in serum from the subject indicates the presence of RA.

Preferably, the abundance of an RPI is normalized to an Expression Reference Protein Isoform (ERPI). ERPIs have been identified by partial amino acid sequencing of the ERFs described above, using the methods and apparatus of the Preferred Technology. The partial amino acid sequences of these ERPIs, and the known proteins to which they are homologous are presented in table XIb.

TABLE XIb

ERPI-#	ERF-#	Homologous Protein	Partial Amino Acid Sequences
ERPI-1	ERF-4	Zn-α-2-glycoprotein precursor	EDIFMETLK
ERPI-2	ERF-6	Immunoglobulin heavy chain γ (intermediate segment)	EEQYNSTYR
ERPI-3	ERF-8	α-2-HS-glycoprotein precursor	LDGKFSVVYAK

As shown above, the RPIs described herein include previously unknown proteins, as well as isoforms of known proteins where the isoforms were not previously known to be associated with RA. For each RPI, the present invention additionally provides a preparation comprising the isolated RPI or fragments thereof, and antibodies that bind to said

RPI or to said fragments, or to said RPI and said fragments. As used herein, an "isolated" RPI is an RPI free of proteins or protein isoforms having a significantly different pI or MW from those of the RPI, as determined by 2D electrophoresis. As used herein, a "significantly different" pI or MW is one that permits the contaminating protein isoform to be resolved from the RPI on 2D electrophoresis, performed according to the Reference Protocol.

In one embodiment, an isolated protein is provided, said protein comprising a peptide with the amino acid sequence identified in Table VIII, IX, X or XI for an RPI, said protein having a pI and MW within 10% (preferably within 5%, more preferably within 1%) of the values identified in Tables VIII, IX, X and XI for that RPI.

The RPIs of the invention can be assayed by any method known to those skilled in the art. In one embodiment, the RPIs are separated on a 2-D gel by virtue of their MWs and pIs and visualized by staining the gel.

Alternatively, RPIs can be detected in assays, such as immunoassays, for detection, prognosis, diagnosis, or monitoring of RA or for drug development. In one embodiment, an immunoassay is performed by contacting a sample derived from a subject to be tested with an anti-RPI antibody under conditions such that immunospecific binding can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. Preferably, the anti-RPI antibody preferentially binds to the RPI rather than to other isoforms of the same protein. In a preferred embodiment, the anti-RPI antibody binds to the RPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms of the same protein.

In another embodiment, an immunoassay is performed by contacting a sample derived, for example, from a subject to be tested, with a plurality of anti-RPI antibodies under conditions such that immunospecific binding can occur, and simultaneouslydetecting or measuring the amount of any immunospecific binding by the plurality of antibodies to a plurality of RPIs. Preferably, each anti-RPI antibody binds to a different RPI, and is optionally fixed to a solid support. For example, antibodies can be fixed in a two dimensional array arrangment, wherein each position of the array is occupied by antibodies that specifically bind a single RPI, and wherein the array has antibodies specific for one or more RPIs. Preferably, each anti-RPI antibody preferentially binds to an RPI rather than to other isoforms of the same protein. In a preferred embodiment, the anti-RPI antibodies bind to the RPIs with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms of the same proteins.

In one embodiment, binding of antibody in tissue sections can be used to detect aberrant RPI localization or aberrant (*e.g.*, high, low, absent) levels of an RPI. In a specific embodiment, antibody to an RPI can be used to assay in a patient tissue or serum sample for the presence of the RPI where an aberrant level of RPI is indicative of RA. As used herein, an "aberrant level" means an increased or decreased level relative to that present, or relative to a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having RA.

The immunoassays which can be used include without limitation competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions,

immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few.

If desired, an RPI can be detected by means of a two-step sandwich assay. Where an RPI represents a particular glycoform of a protein, the first step can employ an anti-RPI antibody (which can optionally be immobilized on a solid phase) to capture the RPI; in the second step, a directly or indirectly labelled lectin is used to detect the captured RPI. Any lectin can be used for this purpose that preferentially binds to the RPI rather than (a) to other glycoforms that have the same core protein as the RPI or (b) to other isoforms that share the antigenic determinant recognized by the antibody. In a preferred embodiment, the chosen lectin binds to the RPI with at least 2-fold greater affinity, more preferably at least 5-fold greater affinity, still more preferably at least 10-fold greater affinity, than to said other isoforms that have the same core protein as the RPI. A lectin that is suitable for detecting a given RPI can readily be identified by methods well known in the art, for instance upon testing one or more lectins enumerated in Table I on pages 158-159 of Sumar et al., Lectins as Indicators of Disease-Associated Glycoforms, *In*: Gabius H-J & Gabius S (eds.), 1993, Lectins and Glycobiology, at pp. 158-174 (which is incorporated herein by reference in its entirety).

If desired, a gene encoding an RPI, a related gene and related nucleic acid sequences and subsequences, including complementary sequences, can also be used in hybridization assays. A nucleotide encoding an RPI, or subsequences thereof comprising about at least 8 nucleotides, or the complement of the foregoing can be used as hybridization probes. Hybridization assays can be used for detection,

prognosis, diagnosis, or monitoring of conditions, disorders, or disease states, associated with aberrant changes in RPI gene expression, in particular RA or recrudescence of RA following therapy. In particular, such a hybridization assay is carried out by a method comprising contacting a sample containing nucleic acid with a nucleic acid probe capable of hybridizing to a DNA or RNA encoding an RPI, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization.

The invention also provides diagnostic kits, comprising in one or more containers an anti-RPI antibody. In addition, such a kit may optionally comprise one or more of the following: (1) instructions for using the anti-RPI antibody for diagnosis, prognosis, therapeutic monitoring, drug development or any combination of these applications; (2) a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration; (3) a labeled binding partner to the antibody; and (4) a solid phase (such as a reagent strip) upon which the anti-RPI antibody is immobilized. If no labeled binding partner to the antibody is provided, the anti-RPI antibody itself can be labeled with a detectable marker, *e.g.*, a chemiluminescent, enzymatic, fluorescent, or radioactive moiety.

The invention also provides a kit comprising in one or more containers a nucleic acid probe capable of hybridizing to RNA encoding a distinct RPI. In a specific embodiment, a kit can comprise in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides, more preferrably 10-20 nucleotides) that are capable of priming amplification, -- such as by polymerase chain reaction (see

e.g., Innis et al., 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of Qβ replicase, cyclic probe reaction, or other methods known in the art -- under appropriate reaction conditions of at least a portion of a nucleic acid encoding an RPI.

Kits are also provided which allow for the detection of a plurality of RPIs or a plurality of nucleic acids each encoding an RPI. A kit can optionally further comprise a predetermined amount of an isolated RPI protein or a nucleic acid encoding an RPI, e.g., for use as a standard or control.

5.3 Use in Clinical Studies The diagnostic methods and compositions of the present invention can assist in monitoring a clinical study, e.g., for testing drugs for therapy of RA. In one embodiment, candidate molecules are tested for their ability to restore RADF or RPI levels in a patient suffering from RA towards levels found in subjects not suffering from RA or, in a treated patient to maintain RADF or RFI levels at or near non-RA or serum values. The levels of one or more RADFs or RPIs can be assayed.

In another embodiment, the methods and compositions of the present invention are used to identify individuals with RA when screening candidates for a clinical study; such individuals can then be included in or excluded from the study or can be placed in a separate cohort for treatment or analysis.

5.4 Purification of RPIs In particular aspects, the invention provides isolated RPIs, preferably human RPIs, and fragments and derivatives thereof which comprise an antigenic determinant (i.e., can be recognized by an antibody) or which are otherwise

functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active" RPI as used herein refers to that material displaying one or more known functional activities associated with a full-length (wild-type) RPI, e.g., binding to an RPI substrate or RPI binding partner, antigenicity (binding to an anti-target antibody), immunogenicity, etc.

In specific embodiments, the invention provides fragments of an RPI comprising at least 6 amino acids, 10 amino acids, 50 amino acids, or at least 75 amino acids. Fragments, or proteins comprising fragments, lacking some or all of the regions of an RPI are also provided. Nucleic acids encoding the foregoing are provided.

Once a recombinant nucleic acid which expresses the RPI gene sequence is identified, the gene product can be analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labelling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

Once the RPI is identified, it can be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

Alternatively, once an RPI produced by a recombinant nucleic acid is identified, the entire amino acid sequence of the RPI can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant. As a result, the protein can be synthesized by standard chemical methods known in the art (e.g., see Hunkapille, et al., M., 1984, *Nature* 310:105-111).

In another alternative embodiment, native RPIs can be purified from natural sources, by standard methods such as those described above (e.g., immunoaffinity purification).

In a preferred embodiment, RPIs are isolated by the Preferred Technology described in U.S. Application No. 08/980,574, which is incorporated herein by reference. For preparative-scale runs, a narrow-range "zoom gel" having a pH range of 2 pH units or less is preferred for the isoelectric step, according to the method described in Westermeier, 1993, Electrophoresis in Practice (VCH, Weinheim, Germany), pp. 197-209 (which is incorporated herein by reference in its entirety); this modification permits a larger quantity of a target protein to be loaded onto the gel, and thereby increases the quantity of isolated RPI that can be recovered from the gel. When used in this way for preparative-scale runs, the Preferred Technology typically provides up to 100 ng, and can provide up to 1000 ng, of an isolated RPI in a single run. Those of skill in the art will appreciate that a zoom gel can be used in any separation strategy which employs gel isoelectric focusing.

In a specific embodiment of the present invention, such RPIs, whether produced by recombinant DNA techniques or by chemical synthetic methods or by purification of native proteins, include (but are not limited to) those containing all or part of the amino acid sequence of the RPI, as well as fragments and other derivatives, and analogs thereof, including proteins homologous thereto.

5.5 Production of Antibodies to RPIs According to the invention, an RPI, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such

proteins, fragments, derivatives, or analogs can be isolated by any convenient means, including the methods described in the preceding section of this application. The antibodies generated include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to a human RPI are produced. In another embodiment, antibodies to a domain of an RPI are produced. In a specific embodiment, hydrophilic fragments of an RPI are used as immunogens for antibody production.

1.1

Various procedures known in the art may be used for the production of polyclonal antibodies to an RPI or derivative or analog. In a particular embodiment, rabbit polyclonal antibodies to an epitope of an RPI, or a subsequence thereof, can be obtained. For the production of antibody, various host animals can be immunized by injection with the native RPI, or a synthetic version, or derivative (e.g., fragment) thereof, including but not limited to rabbits, mice, rats, horses, goats etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to complete or incomplete Freund's adjuvant, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

For preparation of monoclonal antibodies directed toward an RPI sequence or analog thereof, any technique which

provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). (Each of the foregoing references is incorporated herein by reference.) In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals as described in PCT/US90/02545, which is incorporated herein by reference. According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Neuberger et al., 1984, *Nature* 312:604-608; Takeda et al., 1985, *Nature* 314:452-454) by splicing the genes from a mouse antibody molecule specific for an RPI together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. (Each of the foregoing references is incorporated herein by reference.)

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778, incorporated herein by reference) can be adapted to produce RPI-specific single-chain antibodies. An additional

embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, *Science* 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for RPIs, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of an RPI, one may assay generated hybridomas for a product which binds to an RPI fragment containing such domain. For selection of an antibody that specifically binds a first RPI homolog but which does not specifically bind a different RPI homolog, one can select on the basis of positive binding to the first RPI homolog and a lack of binding to the second RPI homolog. Similarly, for selection of an antibody that specifically binds an RPI but which does not specifically bind a different isoform of the same protein (*e.g.*, a different glycoform having the same core peptide as the RPI), one can select on the basis of positive binding to the RPI and a lack of binding to the different isoform (*e.g.*, glycoform).

In addition, techniques developed for the production of "chimeric antibodies" (Morrison, et al., 1984, Proc

. Natl. Acad.Sci., 81, 6851-6855; Neuberger, et al., 1984, Nature 312, 604-608; Takeda, et al., 1985, Nature, 314, 452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity c an be used. A chimeric antibody is a molecule in whi ch different portions are derived from different anim al species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. (See, e.g., Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U .S. Patent No. 4,816397, which are incorporated herein by reference in their entirety.)

In addition, techniques have been developed for the production of humanized antibodies. (See, e.g., Queen, U.S Patent No. 5,585,089 and Winter, U.S. Pa tent No. 5,225,539, which are incorporated herein by reference in their entirety.) An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, referred to as complementarity determining regions (CDRs). The extent of the framework region and CDRs have been precisely defined (see, "Sequences of Proteins of Immunological Interest", Kabat, E. et al., U.S.Depart ment of Health and Human Services (1983)). Briefly, humanized antibodies are antibody molecules from no n-human species having one or more CDRs from the n on-human species and a framework region from a human immunoglobulin molecule.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4, 946,778; Bird, 1988, Science 242, 423-426; Huston, e t al., 1988, Proc. Natl. Acad. Sci. USA 85, 5879-588 3; and Ward, et al., 1989, Nature 334, 544-546) can be used. Single chain antibodies are formed by linki ng the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize specific epitopes may begenerated by known techniques. For

example, such fragments include but are not limited to: the F(ab')2 fragments, which can be produced by pepsin digestion of the antibody molecule and the Fab fragments, which can be generated by reducing the disulfide bridges of the F(ab')2 fragments.

Alternatively, Fab expression libraries may be constructed (Huse, et al., 1989, Science, 246, 1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

LABELING OF ANTIBODIES AND USES THEREOF

Described herein are methods for detectably labeling molecules capable of specifically recognizing one or more RPI epitopes or epitopes of conserved variants or peptide fragments of an RPI. The labeling and detection methods employed herein

may, for example, be such as those described in Harlow and Lane (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual". Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), which is incorporated herein by reference in its entirety.

One of the ways in which the RPI-specific antibody or peptide mimetic can be labeled is by linking the same to an enzyme, such labeled molecules can be used in an enzyme immunoassay such as ELISA (enzyme linked immunosorbent assay). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibodies, derivatives and analogs thereof, and peptides include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcoholdehydrogenase, alpha-glycerophosphate, dehydrogenase, triosephosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-

phosphate-dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods whichemploy a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison withsimilarly prepared standards.

For use in detection methods, the molecules are preferably labeled with a radioisotope, including but not limited to: 125I, 131I, or 99mTc. Such peptides and antibodies can be detected in in vivo assays using a radioimmunoassay (RIA) or radioprobe. The radioactive isotope can be detected by such means asthe use of agamma counter or a scintillation counter or by autoradiography.It is also possible to label the antibodies, derivatives andanalogs thereof, and peptides with a fluorescent compound. When the fluorescently labeled peptide is exposed to light of theproper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

It is also possible to label the antibodies, derivatives and analogs thereof, and peptides with biotin. The biotin labeled peptide can be exposed to an avidin-conjugated detectable marker, such as a fluorescent label conjugated to avidin. Because avidin binds with high affinity to biotin, the avidin-conjugated detectable marker becomes associated with the biotin labeled peptide, thereby allowing for the detection of the peptide.

The antibodies, derivatives and analogs thereof, andpeptides can also be detectably labeled using fluorescenceemitting metals such as 152Eu, or others of the lanthanide series. These metals can be attached to the antibodies, derivatives andanalogs thereof, and peptides using such metal chelating groupsas diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibodies, derivatives and analogs thereof, and peptides also can be detectably labeled by coupling to achemiluminescent compound. The presence of the chemiluminescent-tagged peptides are

then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label theantibodies, derivatives and analogs thereof, and peptides of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which acatalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence ofluminescence. Important bioluminescent compounds for purposes of labeling areluciferin, luciferase and aequorin.

The labeled antibodies that are determined to specifically bind an RPI can be used to detect the presence of the RPI in a variety of biological samples, including a body fluid (e.g.,plasma, urine, cerebrospinal fluid, joint aspirate), a tissue sample, or homogenate thereof. Such labeled antibodies may also be used to visualize the localization of RPIs within or on individual cells.

The labeled antibodies that are determined to specifically bind an RPI can be administered to a patient at diagnostically

effective doses to detect the presence of an RPI. A

diagnostically effective dose refers to that amount of the

molecule sufficient to target a diagnostic to a cell containing

an RPI on its surface such that the cell can be detected using

methods commonly available in the art.

Antibodies specific to a domain of an RPI are also provided.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the RPIs of the invention, e.g., for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.

5.6 Isolation Of DNA Encoding An RPI Specific embodiments for the cloning of an RPI gene, are presented below by way of example and not of limitation.

The nucleotide sequences of the present invention, including DNA and RNA, and comprising a sequence encoding the RPI or a fragment or analog thereof, may be synthesized using methods known in the art, such as using conventional chemical approaches or polymerase chain reaction (PCR) amplification of overlapping oligonucleotides. The sequences also provide for the identification and cloning of the RPI gene from any species, for instance for screening cDNA libraries, genomic libraries or expression libraries.

The nucleotide sequences comprising a sequence encoding an RPI of the present invention are useful for their ability to selectively form duplex molecules with complementary stretches of other protein genes. Depending on the application, a variety of hybridization conditions may be employed to achieve varying sequence identities.

For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt or high temperature conditions. As used herein, "highly stringent conditions" means hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et

al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3; incorporated herein by reference in its entirety.) For some applications, less stringent hybridization conditions are required. As used herein "moderately stringent conditions" means washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*). Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be chosen depending on the desired results. For example, convenient hybridization temperatures in the presence of 50% formamide are: 42°C for a probe which is 95 to 100% homologous to the RPI gene fragment, 37°C for 90 to 95% homology and 32°C for 70 to 90% homology.

In the preparation of genomic libraries, DNA fragments are generated, some of which will encode a part or the whole of an RPI. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis, column chromatography and sucrose gradient centrifugation. The DNA fragments can then be inserted into suitable vectors, including but not limited to plasmids, cosmids, bacteriophages lambda or T_4, and yeast artificial chromosome (YAC). (See, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M.

(ed. , 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd. Oxford, U.K. Vol. I, II; Ausubel F.M. et al., eds., 1989 Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York . The genomic library may be screened by nucleic acid hybridization to labeled probe (Benton and Davis, 1977, *Science* 196:180; Grunstein and Hogness, 1975, *Proc. Natl. Acad. Sci. USA* 72:3961).

The genomic libraries may be screened with labeled degenerate oligonucleotide probes corresponding to the amino acid sequence of any peptide of the RPI using optimal approaches well known in the art. Any probe used preferably is 10 nucleotides or longer, more preferably 15 nucleotides or longer.

As shown in Tables VIII to XI above, some RPIs disclosed herein correspond to previously identified proteins encoded by genes whose sequences are publicly known. To screen such a gene, any probe may be used that is complementary to the gene or its complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. The Entrez database held by the National Center for Biotechnology Information (NCBI) -- which is accessible at *http://www.ncbi.nlm.nih.gov/* -- provides gene sequences for these RPIs under the following accession numbers, and each sequence is incorporated herein by reference:

Table XII. Gene sequences of RPI-related proteins

RADF #	RPI	Accession numbers
RADF-1	RPI-1	T40090, T40068
RADF-3	RPI-3	AA551927, AA260531, W97741, N99366, T70526, T40177, T40060, T40034, T39910, T39894

RADF-4	RPI-4	T41010, T40102, T40058, T39954
RADF-6	RPI-5	AA269874
RADF-8	RPI-6	Z20858, AA503766, H51308, H03365
RADF-9	RPI-7	Z20858, AA503766, H51308, H03365
RADF-10	RPI-8	Z21022, Z19947
RADF-11	RPI-9	T41063, T41020, T41005, T40881, T40190, T40139, T40125, T40114, T40096, T39908
RADF-12	RPI-11	T40090, T40068
RADF-13	RPI-12	AA551927, AA260531, W97741, N99366, T70526, T40177, T40060, T40034, T39910, T39894
RADF-14	RPI-13	T40090, T40068
RADF-15	RPI-15	T40940, T40002
RADF-16	RPI-16	T73244, T71043, T71032, T40181, T40116
RADF-18	RPI-21	N99641, N99445, N99528, Z20485, Z20465
RADF-19	RPI-22	AA269874
RADF-20	RPI-23	Z20858, AA503766, H51308, H03365
RADF-22	RPI-24	T1952, H73939, Z20894, T40182, T40167, T40158
RADF-23	RPI-25	AA614684, AA523377, AA715907, AA580429, AA630254, AA617854, AA580356
RADF-24	RPI-26	AA268201, T64416, T62149, T40186
RADF-25	RPI-27	Z21017, Z20888, Z19984, Z19971, T41056, T40108
RADF-26	RPI-28	T1952, H73939, Z20894, T40182, T40167, T40158
RADF-26	RPI-29	Z21017, Z20888, Z19984, Z19971, T41056, T40108
RADF-26	RPI-30	T64707
RADF-27	RPI-31	Z20858, AA503766, H51308, H03365
RADF-32	RPI-33	T40090, T40068
RADF-33	RPI-34	T40090, T40068
RADF-35	RPI-36	Z21022, Z19947

RADF-36	RPI-37	C20858 AA503765, H51308, H03365

For each of RPI-10, RPI-17, and RPI-20, a degenerate set of probes is provided, as follows:

(a) Probes for RPI-10

```
5'- A C G A C C T T T A T G T T C T A T A G C A G C A A C -3'
    G   T G     G     G     C     C   G G     G     G
    T     T                       A   A T     T     T
    C     C                       G     C     C     C
```

(b) Probes for RPI-17

```
5'- A C G A G C A T G A G G A G C A T C T A T A A G A T T -3'
    G   T G     G     G     G     G     C   G G     G
    T     T           T     T           A   A T
    C     C           C     C           G     C
```

(c) Probes for RPI-20

```
5'- A G A T A T A T C A G C A C C A G A A T C -3'
    G C T C   G G     G     G     G C T G
    T     A   A       T     T     T
    C     G           C     C     C
```

Clones in libraries with insert DNA encoding the RPI or fragments thereof will hybridize to one or more of the degenerate oligonucleotide probes (or their complement). Hybridization of such oligonucleotide probes to genomic libraries are carried out using methods known in the art. For example, hybridization with one of the above-mentioned degenerate sets of oligonucleotide probes, or their complement (or with any member of such a set, or its complement) can be performed under highly stringent or moderately stringent conditions as defined above, or can be carried out in 2X SSC, 1.0% SDS at 50° C and washed using the same conditions.

In yet another aspect clones of nucleotide sequences encoding a part or the entire RPI or RPI-derived polypeptides may also be obtained by screening expression libraries. For example, DNA from the relevant source is isolated and random fragments are prepared and ligated into an expression vector (*e.g.*, a bacteriophage, plasmid, phagemid or cosmid) such that the inserted sequence in the vector is capable of being expressed by the host cell into which the vector is then introduced. Various screening assays can then be used to select for the expressed RPI or RPI-derived polypeptides. In one embodiment, the various anti-RPI antibodies of the invention can be used to identify the desired clones using methods known in the art. See, for example, Harlow and Lane, 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Appendix IV. Clones or plaques from the library are brought into contact with the antibodies to identify those clones that bind.

In an embodiment, colonies or plaques containing DNA that encodes an RPI or RPI-derived polypeptide can be detected using DYNA Beads according to Olsvick et al., 29th ICAAC, Houston, Tex. 1989, incorporated herein by reference. Anti-RPI antibodies are crosslinked to tosylated DYNA Beads M280, and these antibody-containing beads would then be used to adsorb to colonies or plaques expressing RPI or RPI-derived polypeptide. Colonies or plaques expressing an RPI or RPI-derived polypeptide are identified as any of those that bind the beads.

Alternatively, the anti-RPI antibodies can be nonspecifically immobilized to a suitable support, such as silica or Celite™ resin. This material would then be used to adsorb to bacterial colonies expressing the RPI protein or

RPI-derived polypeptide as described in the preceding paragraph.

In another aspect, PCR amplification may be used to produce substantially pure DNA encoding a part of or the whole of an RPI from genomic DNA. Oligonucleotide primers, degenerate or otherwise, corresponding to known RPI sequences can be used as primers.

PCR can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp™). One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the degenerate primers and the corresponding sequences in the DNA. After successful amplification of a segment of the sequence encoding an RPI, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete genomic clone. This, in turn, will permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its protein product for functional analysis, as described *infra*.

The RPI gene can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified RPI DNA of another species (*e.g.*, mouse, human). Immunoprecipitation analysis or functional assays (*e.g.*, aggregation ability *in vitro*; binding to receptor) of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that

contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against an RPI. A radiolabelled RPI cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe to identify the RPI DNA fragments from among other genomic DNA fragments.

Alternatives to isolating RPI genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the RPI. For example, RNA for cDNA cloning of the RPI gene can be isolated from cells which express the RPI. Other methods are possible and within the scope of the invention.

Any eukaryotic cell potentially can serve as the nucleic acid source for the molecular cloning of the RPI gene. The nucleic acid sequences encoding the RPI can be isolated from vertebrate, mammalian, human, porcine, bovine, feline, avian, equine, canine, as well as additional primate sources, insects, plants, etc. The DNA may be obtained by standard procedures known in the art from cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (See, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source,

the RPI gene should be molecularly cloned into a suitable vector for propagation.

The identified and isolated gene or cDNA can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and RPI gene may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated RPI gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the

transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The RPI sequences provided by the instant invention include those nucleotide sequences encoding substantially the same amino acid sequences as found in native RPIs, and those encoded amino acid sequences with functionally equivalent amino acids, as well as those encoding other target derivatives or analogs.

5.7 Expression of DNA Encoding an RPI The nucleotide sequence encoding an RPI or a functionally active analog or fragment or other derivative thereof can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native RPI gene or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, the human RPI gene is expressed, or a sequence encoding a

functionally active portion of the human RPI. In yet another embodiment, a fragment of target comprising a domain of the RPI is expressed.

1.1

1.1

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional and translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding an RPI or peptide fragment may be regulated by a second nucleic acid sequence so that the RPI or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of an RPI may be controlled by any promoter or enhancer element known in the art. Promoters which may be used to control RPI gene expression include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff et al., 1978, *Proc. Natl. Acad. Sci. USA* 75:3727-3731), or the *tac* promoter (DeBoer et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:21-25); see also "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; plant expression vectors comprising the

nopaline synthetase promoter region (Herrera-Estrella et al., *Nature* 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, *Nucl. Acids Res.* 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, *Nature* 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, *Cell* 38:639-646; Ornitz et al., 1986, *Cold Spring Harbor Symp. Quant. Biol.* 50:399-409; MacDonald, 1987, *Hepatology* 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, *Nature* 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, *Cell* 38:647-658; Adames et al., 1985, *Nature* 318:533-538; Alexander et al., 1987, *Mol. Cell. Biol.* 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, *Cell* 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, *Genes and Devel.* 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, *Mol. Cell. Biol.* 5:1639-1648; Hammer et al., 1987, *Science* 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, *Genes and Devel.* 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, *Nature* 315:338-340; Kollias et al., 1986, *Cell* 46:89-94; myelin basic protein gene control region

which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, *Science* 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to an RPI-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning an RPI coding sequence into the *Eco*RI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; Smith and Johnson, 1988, *Gene* 7:31-40). This allows for the expression of the RPI product from the subclone in the correct reading frame.

Expression vectors containing RPI gene inserts can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of an RPI gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted RPI gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of an RPI gene in the vector. For example, if the

RPI gene is inserted within the marker gene sequence of the vector, recombinants containing the RPI gene insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the RPI gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the RPI in *in vitro* assay systems, *e.g.*, binding with anti-RPI antibody.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (*e.g.*, lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered RPI may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (*e.g.*, glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can

be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the differentially expressed or pathway gene protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the differentially expressed or pathway gene protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the differentially expressed or pathway gene protein.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc*

Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, *Cell* 22:817) genes can be employed in tk⁻, hgprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980, *Natl. Acad. Sci. USA* 77:3567; O'Hare et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, *J. Mol. Biol.* 150:1); and hygro, which confers resistance to hygromycin (Santerre et al., 1984, *Gene* 30:147) genes.

In other specific embodiments, the RPI, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence (of a different protein)). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

5.8 Therapeutic Use Of RPIs The invention provides for treatment or prevention of various diseases and disorders by administration of a therapeutic compound. Such compounds include but are not limited to: RPIs and analogs and derivatives

including fragments thereof (e.g., as described herein); antibodies thereto (as described herein); nucleic acids encoding RPIs, analogs, or derivatives (e.g., as described herein); RPI gene antisense nucleic acids, and RPI gene agonists and antagonists. As is described herein, an important feature of the present invention is the identification of RPI genes involved in rheumatoid arthritis. Arthritis can be treated or prevented by administration of a therapeutic compound that promotes function or expression of RPIs which are decreased in synovial fluid verses serum of RA patients. Arthritis can also be treated or prevented by administration of a therapeutic compound that reduces function or expression of RPIs which are increased in synovial fluid verses serum of RA patients.

Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, a human RPI, derivative, or analog, or nucleic acid, or an antibody to a human RPI, is administered to a human patient for therapy or prophylaxis.

5.8.1 Treatment And Prevention Of Rheumatoid Arthritis

Rheumatoid arthritis is treated or prevented by administration of a compound that promotes (*i.e.*, increases or supplies) the level or function of one or more RPIs -- or the level of one or more RADFs -- that are decreased in synovial fluid verses serum of subjects with RA. Examples of such a compound include but are not limited to RPIs, derivatives, or fragments that are functionally active,

particularly that are active as demonstrated in *in vitro* assays or in animal models, and nucleic acids encoding an RPI or functionally active derivative or fragment thereof (*e.g.*, for use in gene therapy). Other compounds that can be used, *e.g.*, RPI agonists, can be identified using *in vitro* assays.

Rheumatoid arthritis is also treated or prevented by administration of a compound that inhibits (*i.e.*, decreases) the level or function of one or more RPIs -- or the level of one or more RADFs -- that exhibit increased abundance in synovial fluid verses serum of subjects with RA. Examples of such a compound include but are not limited to RPI anti-sense oligonucleotides, ribozymes, or antibodies directed against RPIs. Other compounds that can be used, *e.g.*, RPI antagonists, can be identified using *in vitro* assays.

In specific embodiments, compounds that promote the level or function of one or more RPIs, or the level of one or more RADFs are administered therapeutically (including prophylactically when an absent or decreased (relative to normal or desired) RPI level or function, or RADF level has been identified in synovial fluid of RA patients as compared with serum in RA patients. In further embodiments, compounds that inhibit RPI level or function, or RADF level are administered therapeutically (including prophylactically when an increased (relative to normal or desired) RPI level or function, or RADF level has been identified in synovial fluid of RA patients as compared with serum in RA patients. The change in RPI function or level, or RADF level due to the administration of such compounds can be readily detected, *e.g.*, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or protein levels, or activity of the expressed RPI RNA or protein. The Preferred Technology can also be used to detect levels of the

RPI or RADF before and after the administration of the compound. Many methods standard in the art can be thus employed, including but not limited to kinase assays, immunoassays to detect and/or visualize the RPI (e.g., Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect RPI expression by detecting and/or visualizing mRNA encoding the RPI (e.g., Northern assays, dot blots, *in situ* hybridization, etc.), etc.

The compounds of the invention include but are not limited to any compound, e.g., a small organic molecule, protein, peptide, antibody, nucleic acid, etc. that restores the RA RPI or RADF profile towards normal with the proviso that such compound is not a non-steroidal anti-inflammatory agent (NSAID) (e.g. prednisone, ibuprofen, fenoprofen, ketoprofen, flurbiprofen, indomethacin, sulindac, aspirin, salicylsalicylic acid, diflunisal, naproxen, piroxicam, tenoxicam, phenylbutazone, oxyphenbutazone), a gold salt, D-penicillamine, an antimalarial such as hydroxychloroquine or sulphasalazine, azathioprine, cyclophosphamide, chlorambucil, methotrexate, a corticosteroid, anti-CD4 monoclonal antibody, or anti-CDw52 antibody.

5.8.2 Gene Therapy In a specific embodiment, nucleic acids comprising a sequence encoding an RPI or functional derivative thereof, are administered to promote RPI function, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acid produces its encoded protein

that mediates a therapeutic effect by promoting RPI function.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, *Clinical Pharmacy* 12:488-505; Wu and Wu, 1991, *Biotherapy* 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; and Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62:191-217; May, 1993, TIBTECH 11(5):155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the compound comprises a nucleic acid encoding an RPI, said nucleic acid being part of an expression vector that expresses an RPI or fragment or chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the RPI coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the RPI coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the RPI nucleic acid (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed

to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Clarke et al.), WO

93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).

In a specific embodiment, a viral vector that contains a nucleic acid encoding an RPI is used. For example, a retroviral vector can be used (see Miller et al., 1993, *Meth. Enzymol.* 217:581-599). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The nucleic acid encoding the RPI to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., 1994, *Biotherapy* 6:291-302, which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, *J. Clin. Invest.* 93:644-651; Kiem et al., 1994, *Blood* 83:1467-1473; Salmons and Gunzberg, 1993, *Human Gene Therapy* 4:129-141; and Grossman and Wilson, 1993, *Curr. Opin. in Genetics and Devel.* 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the

advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, *Current Opinion in Genetics and Development* 3:499-503 present a review of adenovirus-based gene therapy. Bout et al., 1994, *Human Gene Therapy* 5:3-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, *Science* 252:431-434; Rosenfeld et al., 1992, *Cell* 68:143-155; Mastrangeli et al., 1993, *J. Clin. Invest.* 91:225-234; PCT Publication WO94/12649; and Wang, et al., 1995, *Gene Therapy* 2:775-783.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, *Proc. Soc. Exp. Biol. Med.* 204:289-300; U.S. Patent No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction

of foreign genes into cells (see, *e.g.*, Loeffler and Behr, 1993, *Meth. Enzymol.* 217:599-618; Cohen et al., 1993, *Meth. Enzymol.* 217:618-644; Cline, 1985, *Pharmac. Ther.* 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. In a preferred embodiment, epithelial cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the patient. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, a nucleic acid encoding an RPI is introduced into the cells such that it is expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598, dated April 28, 1994; Stemple and Anderson, 1992, *Cell* 71:973-985; Rheinwald, 1980, *Meth. Cell Bio.* 21A:229; and Pittelkow and Scott, 1986, *Mayo Clinic Proc.* 61:771).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

5.8.3 Inhibition Of RPIs To Treat Rheumatoid Arthritis

1.1.1

In one embodiment of the invention, RA is treated or prevented by administration of a compound that antagonizes (inhibits) the RPI levels and/or function of RPIs which are elevated in synovial fluid compared with serum of patients suffering from RA. Compounds that can be used include but are not limited to anti-RPI antibodies (and fragments and derivatives thereof containing the binding region thereof), RPI antisense or ribozyme nucleic acids, and nucleic acids encoding dysfunctional RPIs that are used to "knockout" endogenous RPI function by homologous recombination (see,

e.g., Capecchi, 1989, *Science* 244:1288-1292). In a specific embodiment of the invention, a nucleic acid containing a portion of an RPI gene in which nucleotides encoding an RPI flank (are both 5' and 3' to) a different gene sequence, is used, as an RPI antagonist, to promote RPI inactivation by homologous recombination (see also Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438). Other compounds that inhibit RPI function can be identified by use of known convenient *in vitro* assays, *e.g.*, based on their ability to inhibit binding of RPIs to another protein, or inhibit any known RPI function, as preferably assayed *in vitro* or in cell culture, although genetic assays may also be employed. The Preferred Technology can also be useful for detecting levels of the RPI before and after the administration of the compound. Preferably, suitable *in vitro* or *in vivo* assays, are utilized to determine the effect of a specific compound and whether its administration is indicated for treatment of the affected tissue.

In specific embodiments, compounds that inhibit RPI function are administered therapeutically (including prophylactically) when an increased level of an RPI or RPI function (e.g. greater than the normal level or desired level) is detected in synovial fluid compared with serum of a patient suffering from RA. The increased levels in RPI or function can be readily detected, *e.g.*, by quantifying protein and/or RNA, by obtaining patient synovial fluid and serum samples and assaying them *in vitro* for RNA or protein levels, structure and/or activity of the expressed RNA encoding the RPI, or the RPI itself. Many methods standard in the art can be thus employed, including but not limited to kinase assays, immunoassays to detect and/or visualize RPI

(e.g., Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect RPI expression by detecting and/or visualizing respectively mRNA encoding the RPI (e.g., Northern assays, dot blots, *in situ* hybridization, etc.), etc.

5.8.4 Antisense Regulation of RPIs

1.1.1

In a specific embodiment, RPI function is inhibited by use of RPI antisense nucleic acids. The present invention provides the therapeutic or prophylactic use of nucleic acids comprising at least six nucleotides that are antisense to a gene or cDNA encoding an RPI or a portion thereof. As used herein, an RPI "antisense" nucleic acid refers to a nucleic acid capable of hybridizing to a portion of an RNA encoding an RPI (preferably mRNA) by virtue of some sequence complementarity. The antisense nucleic acid may be complementary to a coding and/or noncoding region of an mRNA encoding an RPI. Such antisense nucleic acids have utility as compounds that inhibit RPI function, and can be used in the treatment or prevention of rheumatoid arthritis.

The antisense nucleic acids of the invention can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

The invention further provides pharmaceutical compositions comprising an effective amount of the RPI antisense nucleic acids of the invention in a pharmaceutically acceptable carrier, as described *infra*.

In another embodiment, the invention is directed to methods for inhibiting the expression of an RPI nucleic acid

sequence in a prokaryotic or eukaryotic cell comprising providing the cell with an effective amount of a composition comprising an RPI antisense nucleic acid of the invention.

RPI antisense nucleic acids and their uses are described in detail below.

RPI Antisense Nucleic Acids The RPI antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 50 oligonucleotides). In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:6553-6556; Lemaitre et al., 1987, *Proc. Natl. Acad. Sci.* 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988) or blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, *e.g.*, Krol et al., 1988, *BioTechniques* 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, *Pharm. Res.* 5:539-549).

In a preferred aspect of the invention, an RPI antisense oligonucleotide is provided, preferably of single-stranded DNA. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

The RPI antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group

including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

In another embodiment, the oligonucleotide comprises at least one modified sugar moiety, e.g. a sugar moiety selected from the group consisting of arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothicate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run

parallel to each other (Gautier et al., 1987, *Nucl. Acids Res.* 15:6625-6641).

The oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, *Nucl. Acids Res.* 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:7448-7451), etc.

In a specific embodiment, the RPI antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the RPI antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the RPI antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Such

promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, *Nature* 296:39-42), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of an RPI gene, preferably a human RPI gene. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded RPI antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA encoding an RPI it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

5.8.5 Therapeutic Use Of RPI Antisense Nucleic Acids

The RPI antisense nucleic acids can be used to treat (or prevent) rheumatoid arthritis when the RPI of interest is overexpressed in the synovial fluid patients suffering from

rheumatoid arthritis. In a preferred embodiment, a single-stranded DNA antisense RPI oligonucleotide is used.

Cell types which express or overexpress RNA encoding an RPI can be identified by various methods known in the art. Such cell types include but are not limited to leukocytes (*e.g.*, neutrophils, macrophages, monocytes) and resident cells (*e.g.*, synoviocytes). Such methods include, but are not limited to, hybridization with an RPI-specific nucleic acid (*e.g.*, by Northern hybridization, dot blot hybridization, *in situ* hybridization), observing the ability of RNA from the cell type to be translated *in vitro* into an RPI, immunoassay, etc. In a preferred aspect, primary tissue from a patient can be assayed for RPI expression prior to treatment, *e.g.*, by immunocytochemistry or *in situ* hybridization.

Pharmaceutical compositions of the invention, comprising an effective amount of an RPI antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a patient having rheumatoid arthritis.

The amount of RPI antisense nucleic acid which will be effective in the treatment of RA can be determined by standard clinical techniques. Where possible, it is desirable to determine the antisense cytotoxicity of the tumor type to be treated *in vitro*, and then in useful animal model systems prior to testing and use in humans.

In a specific embodiment, pharmaceutical compositions comprising RPI antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the RPI antisense nucleic acids. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to

specific identifiable tumor antigens (Lecnetti et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:2448-2451; Renneisen et al., 1990, *J. Biol. Chem.* 265:16337-16342).

5.8.6 Inhibitory Ribozyme And Triple Helix Approaches

In another embodiment, symptoms of RA may be ameliorated by decreasing the level of RPI gene expression and/or RPI gene product activity by using RPI gene sequences in conjunction with well-known gene "knock-out," ribozyme and/or triple helix methods to decrease the level of RPI gene expression. Among the compounds that may exhibit the ability to modulate the activity, expression or synthesis of the RPI gene, including the ability to ameliorate the symptoms of a RA, are ribozyme, and triple helix molecules. Such molecules may be designed to reduce or inhibit either unimpaired, or if appropriate, mutant target gene activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

Ribozyme molecules designed to catalytically cleave RPI gene mRNA transcripts can be used to prevent translation of target gene mRNA and, therefore, expression of RPI gene product. (See, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, *Science* 247:1222-1225).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, Current Biology 4, 469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the

well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, *e.g.*, U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.

While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy mRNAs encoding an RPI, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, *Molecular Biology and Biotechnology: A Comprehensive Desk Reference*, VCH Publishers, New York, (see especially Figure 4, page 833) and in Haseloff and Gerlach, 1988, Nature, 334, 585-591, which is incorporated herein by reference in its entirety.

Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the mRNA encoding the RPI, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one that occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and that has been extensively described by Thomas Cech and collaborators (Zaug, *et al.*, 1984, Science, 224, 574-578; Zaug and Cech, 1986, Science, 231, 470-475; Zaug, *et al.*, 1986, Nature, 324, 429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47, 207-216). The Cech-type ribozymes have an eight

base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the gene encoding the RPI.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g., for improved stability, targeting, etc.) and should be delivered to cells that express the RPI *in vivo*. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous mRNA encoding the RPI and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Endogenous RPI expression can also be reduced by inactivating or "knocking out" the RPI gene or its promoter using targeted homologous recombination (e.g., see Smithies, et al., 1985, *Nature* 317:230-234; Thomas and Capecchi, 1987, *Cell* 51:503-512; Thompson et al., 1989, *Cell* 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional RPI gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous RPI gene (either the coding regions or regulatory regions of the gene encoding the RPI) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic

stem) cells can be used to generate animal offspring with an inactive target gene (*e.g.*, see Thomas and Capecchi, 1987 and Thompson, 1989, *supra*). However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors.

Alternatively, endogenous RPI gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the RPI gene (*i.e.*, the RPI gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the RPI gene in target cells in the body. (See generally, Helene, 1991, Anticancer Drug Des., 6(6), 569-584; Helene, *et al.*, 1992, Ann. N.Y. Acad. Sci., 660, 27-36; and Maher, 1992, Bioassays 14(12), 807-815).

Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC* triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are

located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

In instances wherein the antisense, ribozyme, and/or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal RPI gene alleles that the possibility may arise wherein the concentration of normal RPI gene product present may be lower than is necessary for a normal phenotype. In such cases, to ensure that substantially normal levels of RPI gene activity are maintained, therefore, nucleic acid molecules that encode and express RPI gene polypeptides exhibiting normal RPI gene activity may, be introduced into cells via gene therapy methods that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the RPI gene encodes an extracellular protein, it may be preferable to co-administer normal RPI gene protein in order to maintain the requisite level of target gene activity.

Anti-sense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules,

as discussed above. These include techniques for chemically synthesizing oligodecxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

5.9 Demonstration Of Therapeutic Or Prophylactic Utility

The compounds of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays which can be used to determine whether administration of a specific compound is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed.

Test compounds can be tested for their ability to restore RADF or RPI levels in patients suffering from RA towards levels found in subjects not suffering from RA or to produce similar changes in experimental animal models of RA (*e.g.*, adjuvant arthritis in rats). Compounds able to restore said levels can be used as lead compounds for further drug discovery, or used therapeutically. RADF and RPI expression can be assayed by the Preferred Technology,

immunoassays, gel electrophoresis followed by visualization, or any other method known to those skilled in the art. Such assays can be used to screen candidate drugs or in clinical monitoring or drug development, where abundance of an RADF or RPI can serve as a surrogate marker for clinical disease.

In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a compound has a desired effect upon such cell types.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used.

5.10 Therapeutic/Prophylactic Administration And Compositions

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a compound of the invention. In a preferred aspect, the compound is substantially purified (e.g. substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid are described above; additional appropriate formulations and routes of administration can be selected from among those described hereinbelow.

Various delivery systems are known and can be used to administer a compound of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, *e.g.*, in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous,

non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (see Langer, *Science* 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

In yet another embodiment, the compound can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); see also Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, *i.e.*, the brain, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see *e.g.*, Joliot et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers,

particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder

or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment of RA can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-

response curves derived from in vitro or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

6 EXAMPLE: PROTEINS FROM SERUM AND SYNOVIAL FLUID OF PATIENTS WITH RA

Using the following Reference Protocol, proteins in serum and synovial fluid from patients with rheumatoid arthritis (RA) or from patients without RA (*i.e.*, patients with gout, osteoarthritis, or traumatic synovitis) were separated by isoelectric focusing followed by SDS-PAGE and compared. Each sample was run in duplicate.

6.1 Sample preparation

1.1

A protein assay was carried out on the sample as received (Pierce BCA Cat # 23225). A volume of serum corresponding to 300µg of total protein was aliquoted and an equal volume of 10% (w/v) SDS (Fluka 71729), 2.3% (w/v) dithiothreitol (BDH 443852A) was added. The sample was heated at 95°C for 5 mins, and then allowed to cool to 20°C . 125µl of the following buffer was then added to the sample:

8M urea (BDH 452043w)
4% CHAPS (Sigma C3023)
65mM dithiotheitol (DTT)
2% (v/v) Resolytes 3.5-10 (BDH 44338 2x)

This mixture was vortexed, and centrifuged at 13000 rpm for 5 mins at 15°C, and the supernatant was analyzed by isoelectric focusing.

6.1 Isoelectric Focusing

Use in Clinical Studies The diagnostic methods and compositions of the present invention can assist in monitoring a clinical study, *e.g.*, for testing drugs for therapy of RA. In one embodiment, candidate molecules are tested for their ability to restore RADF or RPI levels in a patient suffering from RA towards levels found in subjects not suffering from RA or, in a treated patient to maintain RADF or RFI levels at or near non-RA or serum values. The levels of one or more RADFs or RPIs can be assayed.In another embodiment, the methods and compositions of the present invention are used to identify individuals with RA when screening candidates for a clinical study; such individuals can then be included in or excluded from the study or can be placed in a separate cohort for treatment or analysis.Purification of RPIs In particular aspects, the invention provides isolated RPIs, preferably human RPIs, and fragments and derivatives thereof which comprise an antigenic determinant (*i.e.*, can be recognized by an antibody) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. Functionallyactive RPI as used herein refers to that material displaying one or more known functional activities associated with a full-length (wild-type) RPI, *e.g.*, binding to an RPI substrate or RPI binding partner, antigenicity (binding to an anti-target antibody), immunogenicity, etc. In specific embodiments, the invention provides fragments of an RPI comprising at least 6 amino acids, 10 amino acids, 50 amino acids, or at least 75 amino acids. Fragments, or proteins comprising fragments, lacking some or all of the regions of an RPI are also provided. Nucleic acids encoding the foregoing are provided.Once a recombinant nucleic acid which expresses the RPI gene sequence is identified, the gene product can be

analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labelling of the product followed by analysis by gel electrophoresis, immunoassay, etc.Once the RPI is identified, it can be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.Alternatively, once an RPI produced by a recombinant nucleic acid is identified, the entire amino acid sequence of the RPI can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant. As a result, the protein can be synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapille, et al., M., 1984, *Nature* 310105-111).In another alternative embodiment, native RPIs can be purified from natural sources, by standard methods such as those described above (*e.g.*, immunoaffinity purification).In a preferred embodiment, RPIs are isolated by the Preferred Technology described in U.S. Application No. 08/980,574, which is incorporated herein by reference. For preparative-scale runs, a narrow-range zoomgel having a pH range of 2 pH units or less is preferred for the isoelectric step, according to the method described in Westermeier, 1993, Electrophoresis in Practice (VCH, Weinheim, Germany), pp. 197-209 (which is incorporated herein by reference in its entirety); this modification permits a larger quantity of a target protein to be loaded onto the gel, and thereby increases the quantity of isolated RPI that can be recovered from the gel. When used in this way for preparative-scale runs, the Preferred Technology typically provides up to 100 ng, and can provide up to 1000 ng, of an isolated RPI in a single run. Those of

skill in the art will appreciate that a zoom gel can be used in any separation strategy which employs gel isoelectric focusing. In a specific embodiment of the present invention, such RPIs, whether produced by recombinant DNA techniques or by chemical synthetic methods or by purification of native proteins, include (but are not limited to) those containing all or part of the amino acid sequence of the RPI, as well as fragments and other derivatives, and analogs thereof, including proteins homologous thereto. Production of Antibodies to RPIs According to the invention, an RPI, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such proteins, fragments, derivatives, or analogs can be isolated by any convenient means, including the methods described in the preceding section of this application. The antibodies generated include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library. In a specific embodiment, antibodies to a human RPI are produced. In another embodiment, antibodies to a domain of an RPI are produced. In a specific embodiment, hydrophilic fragments of an RPI are used as immunogens for antibody production. Various procedures known in the art may be used for the production of polyclonal antibodies to an RPI or derivative or analog. In a particular embodiment, rabbit polyclonal antibodies to an epitope of an RPI, or a subsequence thereof, can be obtained. For the production of antibody, various host animals can be immunized by injection with the native RPI, or a synthetic version, or derivative (e.g., fragment) thereof, including but not limited to rabbits, mice, rats, horses, goats etc. Various adjuvants may be used to increase the immunological

response, depending on the host species, and including but not limited to complete or incomplete Freund's adjuvant, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.For preparation of monoclonal antibodies directed toward an RPI sequence or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256495-497), as well as the tricma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 472), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). (Each of the foregoing references is incorporated herein by reference. In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals as described in PCT/US90/02545, which is incorporated herein by reference. According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, *Proc. Natl. Acad. Sci. USA* 802026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of chimericantibodies (Morrison et al., 1984, *Proc. Natl. Acad. Sci. USA* 816851-6855; Neuberger et al., 1984, *Nature* 312604-608; Takeda et al., 1985, *Nature* 314452-454) by splicing the genes from a mouse

antibody molecule specific for an RPI together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention. (Each of the foregoing references is incorporated herein by reference.)According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778, incorporated herein by reference) can be adapted to produce RPI-specific single-chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, *Science* 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for RPIs, derivatives, or analogs.Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab'. fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.*, ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of an RPI, one may assay generated hybridomas for a product which binds to an RPI fragment containing such domain. For selection of an antibody that specifically binds a first RPI homolog but which does not specifically bind a different RPI homolog, one can select on the basis of positive binding to the first RPI homolog and a lack of binding to the second RPI homolog.

Similarly, for selection of an antibody that specifically binds an RPI but which does not specifically bind a different isoform of the same protein (e.g. a different glycoform having the same core peptide as the RPI), one can select on the basis of positive binding to the RPI and a lack of binding to the different isoform (e.g., glycoform).Antibodies specific to a domain of an RPI are also provided.The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the RPIs of the invention, e.g., for imaging these proteins, measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc.Isolation Of DNA Encoding An RPI Specific embodiments for the cloning of an RPI gene, are presented below by way of example and not of limitation.The nucleotide sequences of the present invention, including DNA and RNA, and comprising a sequence encoding the RPI or a fragment or analog thereof, may be synthesized using methods known in the art, such as using conventional chemical approaches or polymerase chain reaction (PCR) amplification of overlapping oligonucleotides. The sequences also provide for the identification and cloning of the RPI gene from any species, for instance for screening cDNA libraries, genomic libraries or expression libraries.The nucleotide sequences comprising a sequence encoding an RPI of the present invention are useful for their ability to selectively form duplex molecules with complementary stretches of other protein genes. Depending on the application, a variety of hybridization conditions may be employed to achieve varying sequence identities.For a high degree of selectivity, relatively stringent conditions are used to form the duplexes, such as low salt or high temperature conditions. As used herein, highlystringentconditions means hybridization to filter-bound

DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3; incorporated herein by reference in its entirety.) For some applications, less stringent hybridization conditions are required. As used herein moderatelystringentconditions means washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*). Hybridization conditions can also be rendered more stringent by the addition of increasing amounts of formamide, to destabilize the hybrid duplex. Thus, particular hybridization conditions can be readily manipulated, and will generally be chosen depending on the desired results. For example, convenient hybridization temperatures in the presence of 50% formamide are 42°C for a probe which is 95 to 100% homologous to the RPI gene fragment, 37°C for 90 to 95% homology and 32°C for 70 to 90% homology.In the preparation of genomic libraries, DNA fragments are generated, some of which will encode a part or the whole of an RPI. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis, column chromatography and sucrose gradient centrifugation. The DNA fragments can then be inserted into suitable vectors, including but not limited to plasmids, cosmids, bacteriophages lambda or T_4, and yeast artificial chromosome (YAC). (See, for example, Sambrook et al., 1989, Molecular

Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II; Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York). The genomic library may be screened by nucleic acid hybridization to labeled probe (Benton and Davis, 1977, *Science* 196180; Grunstein and Hogness, 1975, *Proc. Natl. Acad. Sci. USA* 723961). The genomic libraries may be screened with labeled degenerate oligonucleotide probes corresponding to the amino acid sequence of any peptide of the RPI using optimal approaches well known in the art. Any probe used preferably is 10 nucleotides or longer, more preferably 15 nucleotides or longer. As shown in Tables VIII to XI above, some RPIs disclosed herein correspond to previously identified proteins encoded by genes whose sequences are publicly known. To screen such a gene, any probe may be used that is complementary to the gene or its complement; preferably the probe is 10 nucleotides or longer, more preferably 15 nucleotides or longer. The Entrez database held by the National Center for Biotechnology Information (NCBI) -- which is accessible at *http//www.ncbi.nlm.nih.gov/* -- provides gene sequences for these RPIs under the following accession numbers, and each sequence is incorporated herein by referenceTable XII. Gene sequences of RPI-related proteinsRADF #RPIAccession numbersRADF-1 RPI-1 T40090, T40068RADF-3 RPI-3 AA551927, AA260531, W97741, N99366, T70526, T40177, T40060, T40034, T39910, T39894RADF-4 RPI-4 T41010, T40102, T40058, T39954RADF-6 RPI-5 AA269874RADF-8 RPI-6 Z20858, AA503766, H51308, H03365RADF-9 RPI-7 Z20858, AA503766,

H51308, H03365RADF-10 RPI-8 Z21022, Z19947RADF-11 RPI-9 T41063, T41020, T41005, T40881, T40190, T40139, T40125, T40114, T40096, T39908RADF-12 RPI-11T40090, T40068RADF-13 RPI-12AA551927, AA260531, W97741, N99366, T70526, T40177, T40060, T40034, T39910, T39894RADF-14 RPI-13T40090, T40068RADF-15 RPI-15T40940, T40002RADF-16 RPI-16T73244, T71043, T71032, T40181, T40116RADF-18 RPI-21N99641, N99445, N99528, Z20485, Z20465RADF-19 RPI-22AA269874RADF-20 RPI-23Z20858, AA503766, H51308, H03365RADF-22 RPI-24T1952, H73939, Z20894, T40182, T40167, T40158RADF-23 RPI-25AA614684, AA523377, AA715907, AA580429, AA630254, AA617854, AA580356RADF-24 RPI-26AA268201, T64416, T62149, T40186RADF-25 RPI-27Z21017, Z20888, Z19984, Z19971, T41056, T40108RADF-26 RPI-28T1952, H73939, Z20894, T40182, T40167, T40158RADF-26 RPI-29Z21017, Z20888, Z19984, Z19971, T41056, T40108RADF-26 RPI-30T64707RADF-27 RPI-31Z20858, AA503766, H51308, H03365RADF-32 RPI-33T40090, T40068RADF-33 RPI-34T40090, T40068RADF-35 RPI-36Z21022, Z19947RADF-36 RPI-37Z20858, AA503766, H51308, H03365For each of RPI-10, RPI-17, and RPI-20, a degenerate set of probes is provided, as follows(a) Probes for RPI-105'- A C G A C C T T T A T G T T C T A T A G C A G C A A C -3' G T G G

G C C G G G G T T

A A T T T C C G

C C C(b) Probes for RPI-175'- A C G A G C A T G A G G A G C A T C T A T A A G A T T -3' G T G G

G G G C G G G T T T

T A A T C C C

C G C (c) Probes for RPI-205'- A G A T A T A T C A G C A C C A G A A T C -3' G C T C G

G G G G C T G T A A T

T T C G C C C

Clones in libraries with insert DNA encoding the RPI or fragments thereof will hybridize to one or more of the degenerate oligonucleotide probes (or their complement). Hybridization of such oligonucleotide probes to genomic libraries are carried out using methods known in the art. For example, hybridization with one of the above-mentioned degenerate sets of oligonucleotide probes, or their complement (or with any member of such a set, or its complement) can be performed under highly stringent or moderately stringent conditions as defined above, or can be carried out in 2X SSC, 1.0% SDS at 50° C and washed using the same conditions.In yet another aspect, clones of nucleotide sequences encoding a part or the entire RPI or RPI-derived polypeptides may also be obtained by screening expression libraries. For example, DNA from the relevant source is isolated and random fragments are prepared and ligated into an expression vector (e.g., a bacteriophage, plasmid, phagemid or cosmid) such that the inserted sequence in the vector is capable of being expressed by the host cell into which the vector is then introduced. Various screening assays can then be used to select for the expressed RPI or RPI-derived polypeptides. In one embodiment, the various anti-RPI antibodies of the invention can be used to identify the desired clones using methods known in the art. See, for example, Harlow and Lane, 1988, Antibodies A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Appendix IV. Clones or plaques from the library are brought into contact with the antibodies to identify those clones that bind.In an embodiment, colonies or plaques containing DNA that encodes an RPI or RPI-derived polypeptide can be detected using DYNA Beads according to Olsvick et al., 29th ICAAC, Houston, Tex. 1989, incorporated herein by

reference. Anti-RPI antibodies are crosslinked to tosylated DYNA Beads M280, and these antibody-containing beads would then be used to adsorb to colonies or plaques expressing RPI or RPI-derived polypeptide. Colonies or plaques expressing an RPI or RPI-derived polypeptide are identified as any of those that bind the beads. Alternatively, the anti-RPI antibodies can be nonspecifically immobilized to a suitable support, such as silica or Celite™ resin. This material would then be used to adsorb to bacterial colonies expressing the RPI protein or RPI-derived polypeptide as described in the preceding paragraph.In another aspect, PCR amplification may be used to produce substantially pure DNA encoding a part of or the whole of an RPI from genomic DNA. Oligonucleotide primers, degenerate or otherwise, corresponding to known RPI sequences can be used as primers.PCR can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp™). One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the degenerate primers and the corresponding sequences in the DNA. After successful amplification of a segment of the sequence encoding an RPI, that segment may be molecularly cloned and sequenced, and utilized as a probe to isolate a complete genomic clone. This, in turn, will permit the determination of the gene's complete nucleotide sequence, the analysis of its expression, and the production of its protein product for functional analysis, as described *infra*.The RPI gene can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to

isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified RPI DNA of another species (e.g., mouse, human). Immunoprecipitation analysis or functional assays (e.g., aggregation ability in vitro; binding to receptor) of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against an RPI. A radiolabelled RPI cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabelled mRNA or cDNA may then be used as a probe to identify the RPI DNA fragments from among other genomic DNA fragments.Alternatives to isolating RPI genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the RPI. For example, RNA for cDNA cloning of the RPI gene can be isolated from cells which express the RPI. Other methods are possible and within the scope of the invention. Any eukaryotic cell potentially can serve as the nucleic acid source for the molecular cloning of the RPI gene. The nucleic acid sequences encoding the RPI can be isolated from vertebrate, mammalian, human, porcine, bovine, feline, avian, equine, canine, as well as additional primate sources, insects, plants, etc. The DNA may be obtained by standard procedures known in the art from cloned DNA (e.g., a DNA library), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (See, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring

Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the RPI gene should be molecularly cloned into a suitable vector for propagation.The identified and isolated gene or cDNA can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and RPI gene may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.In specific embodiments, transformation of host cells with recombinant

DNA molecules that incorporate the isolated RPI gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.The RPI sequences provided by the instant invention include those nucleotide sequences encoding substantially the same amino acid sequences as found in native RPIs, and those encoded amino acid sequences with functionally equivalent amino acids, as well as those encoding other target derivatives or analogs.Expression of DNA Encoding an RPI The nucleotide sequence encoding an RPI or a functionally active analog or fragment or other derivative thereof can be inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native RPI gene or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.*, baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In specific embodiments, the human RPI gene is expressed, or a sequence encoding a functionally

314283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, *Science* 234:1372-1378). In a specific embodiment, a vector is used that comprises a promoter operably linked to an RPI-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (*e.g.*, an antibiotic resistance gene). In a specific embodiment, an expression construct is made by subcloning an RPI coding sequence into the *Eco*RI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; Smith and Johnson, 1988, *Gene* 7:31-40). This allows for the expression of the RPI product from the subclone in the correct reading frame. Expression vectors containing RPI gene inserts can be identified by three general approaches (a) nucleic acid hybridization, (b) presence or absence of marker gene functions, and (c) expression of inserted sequences. In the first approach, the presence of an RPI gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted RPI gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain marker gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of an RPI gene in the vector. For example, if the RPI gene is inserted within the marker gene sequence of the vector, recombinants containing the RPI gene insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the RPI gene product expressed by the recombinant. Such assays can be based, for

example, on the physical or functional properties of the RPI in in vitro assay systems, e.g., binding with anti-RPI antibody.Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered RPI may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure native glycosylation of a heterologous protein. Furthermore, different vector/host expression systems may effect processing reactions to different extents.For long-term,

high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the differentially expressed or pathway gene protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the differentially expressed or pathway gene protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the differentially expressed or pathway gene protein.A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, *Cell* 11223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, *Proc. Natl. Acad. Sci. USA* 482026), and adenine phosphoribosyltransferase (Lowy et al., 1980, *Cell* 22817) genes can be employed in tk$^-$, hgprt$^-$ or aprt$^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980, *Natl. Acad. Sci. USA* 773567; O'Hare et al., 1981, *Proc. Natl. Acad. Sci. USA*

781527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 782072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, *J. Mol. Biol.* 1501); and hygro, which confers resistance to hygromycin (Santerre et al., 1984, *Gene* 30147) genes. In other specific embodiments, the RPI, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence (of a different protein)). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer.Both cDNA and genomic sequences can be cloned and expressed.Therapeutic Use Of RPIs The invention provides for treatment or prevention of various diseases and disorders by administration of a therapeutic compound. Such compounds include but are not limited to RPIs and analogs and derivatives (including fragments) thereof (*e.g.*, as described herein); antibodies thereto (as described herein); nucleic acids encoding RPIs, analogs, or derivatives (*e.g.*, as described herein); RPI gene antisense nucleic acids, and RPI gene agonists and antagonists. As is described herein, an important feature of the present invention is the identification of RPI genes involved in rheumatoid arthritis. Arthritis can be treated or prevented by administration of a therapeutic compound that promotes function or expression of RPIs which are decreased in synovial fluid verses serum of RA

patients. Arthritis can also be treated or prevented by administration of a therapeutic compound that reduces function or expression of RPIs which are increased in synovial fluid verses serum of RA patients.Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, a human RPI, derivative, or analog, or nucleic acid, or an antibody to a human RPI, is administered to a human patient for therapy or prophylaxis.Treatment And Prevention Of Rheumatoid ArthritisRheumatoid arthritis is treated or prevented by administration of a compound that promotes (*i.e.*, increases or supplies) the level or function of one or more RPIs -- or the level of one or more RADFs -- that are decreased in synovial fluid verses serum of subjects with RA. Examples of such a compound include but are not limited to RPIs, derivatives, or fragments that are functionally active, particularly that are active as demonstrated in *in vitro* assays or in animal models, and nucleic acids encoding an RPI or functionally active derivative or fragment thereof (*e.g.*, for use in gene therapy). Other compounds that can be used, *e.g.*, RPI agonists, can be identified using *in vitro* assays.Rheumatoid arthritis is also treated or prevented by administration of a compound that inhibits (*i.e.*, decreases) the level or function of one or more RPIs -- or the level of one or more RADFs -- that exhibit increased abundance in synovial fluid verses serum of subjects with RA. Examples of such a compound include but are not limited to RPI anti-sense oligonucleotides, ribozymes, or antibodies directed against RPIs. Other compounds that can be used, *e.g.*, RPI antagonists, can be identified using *in vitro* assays.In

specific embodiments, compounds that promote the level or function of one or more RPIs, or the level of one or more RADFs are administered therapeutically (including prophylactically when an absent or decreased (relative to normal or desired) RPI level or function, or RADF level has been identified in synovial fluid of RA patients as compared with serum in RA patients. In further embodiments, compounds that inhibit RPI level or function, or RADF level are administered therapeutically (including prophylactically when an increased (relative to normal or desired) RPI level or function, or RADF level has been identified in synovial fluid of RA patients as compared with serum in RA patients. The change in RPI function or level, or RADF level due to the administration of such compounds can be readily detected, *e.g.*, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or protein levels, or activity of the expressed RPI RNA or protein. The Preferred Technology can also be used to detect levels of the RPI or RADF before and after the administration of the compound. Many methods standard in the art can be thus employed, including but not limited to kinase assays, immunoassays to detect and/or visualize the RPI (*e.g.*, Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect RPI expression by detecting and/or visualizing mRNA encoding the RPI (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.), etc.The compounds of the invention include but are not limited to any compound, *e.g.*, a small organic molecule, protein, peptide, antibody, nucleic acid, etc. that restores the RA RPI or RADF profile towards normal with the proviso that such compound is not a non-steroidal

anti-inflammatory agent (NSAID) (e.g. prednisone, ibuprofen, fenoprofen, ketoprofen, flurbiprofen, indomethacin, sulindac, aspirin, salicylsalicylic acid, diflunisal, naproxen, piroxicam, tenoxicam, phenylbutazone, oxyphenbutazone), a gold salt, D-penicillamine, an antimalarial such as hydroxychloroquine or sulphasalazine, azathioprine, cyclophosphamide, chlorambucil, methotrexate, a corticosteroid, anti-CD4 monoclonal antibody, or anti-CDw52 antibody.Gene Therapy In a specific embodiment, nucleic acids comprising a sequence encoding an RPI or functional derivative thereof, are administered to promote RPI function, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acid produces its encoded protein that mediates a therapeutic effect by promoting RPI function.Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, *Clinical Pharmacy* 12488-505; Wu and Wu, 1991, *Biotherapy* 387-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32573-596; Mulligan, 1993, *Science* 260926-932; and Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62191-217; May, 1993, TIBTECH 11(5)155-215). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.In a preferred aspect, the compound comprises a nucleic acid encoding an RPI, said nucleic acid being part of an expression vector that expresses an RPI or fragment or

chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the RPI coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the RPI coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the RPI nucleic acid (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, e.g.,

Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180 dated April 16, 1992 (Wu et al.); WO 92/22635 dated December 23, 1992 (Wilson et al.); WO92/20316 dated November 26, 1992 (Findeis et al.); WO93/14188 dated July 22, 1993 (Clarke et al.), WO 93/20221 dated October 14, 1993 (Young)). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438).In a specific embodiment, a viral vector that contains a nucleic acid encoding an RPI is used. For example, a retroviral vector can be used (see Miller et al., 1993, *Meth. Enzymol.* 217:581-599). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The nucleic acid encoding the RPI to be used in gene therapy is cloned into the vector, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., 1994, *Biotherapy* 6:291-302, which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are Clowes et al., 1994,

J. Clin. Invest. 93644-651; Kiem et al., 1994, *Blood* 83 1467-1473; Salmons and Gunzberg, 1993, *Human Gene Therapy* 4129-141; and Grossman and Wilson, 1993, *Curr. Opin. in Genetics and Devel.* 3110-114.Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, 1993, *Current Opinion in Genetics and Development* 3499-503 present a review of adenovirus-based gene therapy. Bout et al., 1994, *Human Gene Therapy* 53-10 demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., 1991, *Science* 252431-434; Rosenfeld et al., 1992, *Cell* 68143-155; Mastrangeli et al., 1993, *J. Clin. Invest.* 91225-234; PCT Publication WO94/12649; and Wang, et al., 1995, *Gene Therapy* 2775-783.Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., 1993, *Proc. Soc. Exp. Biol. Med.* 204289-300; U.S. Patent No. 5,436,146).Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient. In this embodiment,

the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, *e.g.*, Loeffler and Behr, 1993, *Meth. Enzymol.* 217599-618; Cohen et al., 1993, *Meth. Enzymol.* 217618-644; Cline, 1985, *Pharmac. Ther.* 2969-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.The resulting recombinant cells can be delivered to a patient by various methods known in the art. In a preferred embodiment, epithelial cells are injected, *e.g.*, subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the patient. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes,

macrophages, neutrophils, ecsinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc. In a preferred embodiment, the cell used for gene therapy is autologous to the patient. In an embodiment in which recombinant cells are used in gene therapy, a nucleic acid encoding an RPI is introduced into the cells such that it is expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598, dated April 28, 1994; Stemple and Anderson, 1992, *Cell* 71973-985; Rheinwald, 1980, *Meth. Cell Bio.* 21A229; and Pittelkow and Scott, 1986, *Mayo Clinic Proc.* 61771). In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription. Inhibition Of RPIs To Treat Rheumatoid ArthritisIn one embodiment of the invention, RA is treated or prevented by administration of a compound that antagonizes (inhibits) the RPI levels and/or function of RPIs which are elevated in synovial fluid compared with serum of patients suffering from RA. Compounds that can be used include but are not limited to anti-RPI antibodies (and fragments and derivatives thereof containing the binding region thereof), RPI antisense or ribozyme nucleic acids, and nucleic acids encoding

dysfunctional RPIs that are used to knockout endogenous RPI function by homologous recombination (see, *e.g.*, Capecchi, 1989, *Science* 244:1288-1292). In a specific embodiment of the invention, a nucleic acid containing a portion of an RPI gene in which nucleotides encoding an RPI flank (are both 5' and 3' to) a different gene sequence, is used, as an RPI antagonist, to promote RPI inactivation by homologous recombination (see also Koller and Smithies, 1989, *Proc. Natl. Acad. Sci. USA* 86:8932-8935; Zijlstra et al., 1989, *Nature* 342:435-438). Other compounds that inhibit RPI function can be identified by use of known convenient *in vitro* assays, *e.g.*, based on their ability to inhibit binding of RPIs to another protein, or inhibit any known RPI function, as preferably assayed *in vitro* or in cell culture, although genetic assays may also be employed. The Preferred Technology can also be useful for detecting levels of the RPI before and after the administration of the compound. Preferably, suitable *in vitro* or *in vivo* assays, are utilized to determine the effect of a specific compound and whether its administration is indicated for treatment of the affected tissue.In specific embodiments, compounds that inhibit RPI function are administered therapeutically (including prophylactically) when an increased level of an RPI or RPI function (e.g. greater than the normal level or desired level) is detected in synovial fluid compared with serum of a patient suffering from RA. The increased levels in RPI or function can be readily detected, *e.g.*, by quantifying protein and/or RNA, by obtaining patient synovial fluid and serum samples and assaying them *in vitro* for RNA or protein levels, structure and/or activity of the expressed RNA encoding the RPI, or the RPI itself. Many methods standard in the art can be thus employed, including but not limited to kinase assays,

immunoassays to detect and/or visualize RPI (e.g., Western blot, immunoprecipitation followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and or hybridization assays to detect RPI expression by detecting and/or visualizing respectively mRNA encoding the RPI (e.g. Northern assays, dot blots, in situ hybridization, etc.), etc.Antisense Regulation of RPIsIn a specific embodiment, RPI function is inhibited by use of RPI antisense nucleic acids. The present invention provides the therapeutic or prophylactic use of nucleic acids comprising at least six nuclectides that are antisense to a gene or cDNA encoding an RPI or a portion thereof. As used herein, an RPI antisense nucleic acid refers to a nucleic acid capable of hybridizing to a portion of an RNA encoding an RPI (preferably mRNA) by virtue of some sequence complementarity. The antisense nucleic acid may be complementary to a coding and/or noncoding region of an mRNA encoding an RPI. Such antisense nucleic acids have utility as compounds that inhibit RPI function, and can be used in the treatment or prevention of rheumatoid arthritis.The antisense nucleic acids of the invention can be oligonuclectides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.The invention further provides pharmaceutical compositions comprising an effective amount of the RPI antisense nucleic acids of the invention in a pharmaceutically acceptable carrier, as described *infra*.In another embodiment, the invention is directed to methods for inhibiting the expression of an RPI nucleic acid sequence in a prokaryotic or eukaryotic cell comprising providing the

cell with an effective amount of a composition comprising an RPI antisense nucleic acid of the invention.RPI antisense nucleic acids and their uses are described in detail below.RPI Antisense Nucleic Acids The RPI antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 50 oligonucleotides). In specific aspects, the oligonucleotide is at least 10 nuclectides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger et al., 1989, *Proc. Natl. Acad. Sci. USA* 86:6553-6556; Lemaitre et al., 1987, *Proc. Natl. Acad. Sci.* 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988) or blood-brain barrier (see, *e.g.*, PCT Publication No. WO 89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, *e.g.*, Krol et al., 1988, *BioTechniques* 6:958-976) or intercalating agents (see, *e.g.*, Zon, 1988, *Pharm. Res.* 5:539-549).In a preferred aspect of the invention, an RPI antisense oligonucleotide is provided, preferably of single-stranded DNA. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.The RPI antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil,

5-carboxymethylaminomethyl-2-thiouridine 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.In another embodiment, the oligonucleotide comprises at least one modified sugar moiety, e.g. a sugar moiety selected from the group consisting of arabinose, 2-fluoroarabinose, xylulose, and hexose.In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithicate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et al., 1987, *Nucl. Acids Res.* 15:6625-6641).The oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered

cleavage agent, etc.Oligonucleotides of the invention may be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, *Nucl. Acids Res.* 163209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, *Proc. Natl. Acad. Sci. USA* 857448-7451), etc.In a specific embodiment, the RPI antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the RPI antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the RPI antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to the SV40 early promoter region (Bernoist and Chambon, 1981, *Nature* 290304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, *Cell* 22787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, *Proc. Natl. Acad. Sci. USA* 781441-1445), the regulatory

sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), etc.The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of an RPI gene, preferably a human RPI gene. However, absolute complementarity, although preferred, is not required. A sequence complementarytoatleastaportionofanRNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded RPI antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA encoding an RPI it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.Therapeutic Use Of RPI

Antisense Nucleic AcidsThe RPI antisense nucleic acids can be used to treat (or prevent) rheumatoid arthritis when the RPI of interest is overexpressed in the synovial fluid patients suffering from rheumatoid arthritis. In a preferred embodiment, a single-stranded DNA antisense RPI oligonucleotide is used.Cell types which express or overexpress RNA encoding an RPI can be identified by various methods known in the art. Such cell types include but are not limited to leukocytes (*e.g.*, neutrophils, macrophages, monocytes) and resident cells (*e.g.*, synoviocytes). Such methods include, but are not limited to, hybridization with an RPI-specific nucleic acid (*e.g.*, by Northern

hybridization, dot blot hybridization, *in situ* hybridization), observing the ability of RNA from the cell type to be translated *in vitro* into an RPI, immunoassay, etc. In a preferred aspect, primary tissue from a patient can be assayed for RPI expression prior to treatment, *e.g.*, by immunocytochemistry or *in situ* hybridization.Pharmaceutical compositions of the invention, comprising an effective amount of an RPI antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a patient having rheumatoid arthritis.The amount of RPI antisense nucleic acid which will be effective in the treatment of RA can be determined by standard clinical techniques. Where possible, it is desirable to determine the antisense cytotoxicity of the tumor type to be treated *in vitro*, and then in useful animal model systems prior to testing and use in humans.In a specific embodiment, pharmaceutical compositions comprising RPI antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the RPI antisense nucleic acids. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable tumor antigens (Leonetti et al., 1990, *Proc. Natl. Acad. Sci. USA* 872448-2451; Renneisen et al., 1990, *J. Biol. Chem.* 26516337-16342). Inhibitory Ribozyme And Triple Helix ApproachesIn another embodiment, symptoms of RA may be ameliorated by decreasing the level of RPI gene expression and/or RPI gene product activity by using RPI gene sequences in conjunction with well-known gene knock-out, ribozyme and/or triple helix methods to decrease the level of RPI gene expression. Among the compounds that may exhibit the ability to modulate the activity, expression or synthesis of the RPI

gene, including the ability to ameliorate the symptoms of a RA, are ribozyme, and triple helix molecules. Such molecules may be designed to reduce or inhibit either unimpaired, or if appropriate, mutant target gene activity. Techniques for the production and use of such molecules are well known to those of skill in the art.Ribozyme molecules designed to catalytically cleave RPI gene mRNA transcripts can be used to prevent translation of target gene mRNA and, therefore, expression of RPI gene product. (See, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. (For a review, see Rossi, 1994, Current Biology 4, 469-471). The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage event. The composition of ribozyme molecules must include one or more sequences complementary to the target gene mRNA, and must include the well known catalytic sequence responsible for mRNA cleavage. For this sequence, see, e.g., U.S. Patent No. 5,093,246, which is incorporated herein by reference in its entirety.While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy mRNAs encoding an RPI, the use of hammerhead ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases 5'-UG-3'. The construction and production of hammerhead ribozymes is well known in the art and is described more fully in Myers, 1995, *Molecular Biology and Biotechnology A Comprehensive Desk Reference*,

VCH Publishers, New York, (see especially Figure 4, page 833) and in Haseloff and Gerlach, 1988, Nature, 334, 585-591, which is incorporated herein by reference in its entirety.Preferably the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the mRNA encoding the RPI, *i.e.*, to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.The ribozymes of the present invention also include RNA endoribonucleases (hereinafter Cech-typeribozymes) such as the one that occurs naturally in *Tetrahymena thermophila* (known as the IVS, or L-19 IVS RNA) and that has been extensively described by Thomas Cech and collaborators (Zaug, *et al.*, 1984, Science, 224, 574-578; Zaug and Cech, 1986, Science, 231, 470-475; Zaug, *et al.*, 1986, Nature, 324, 429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986, Cell, 47, 207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present in the gene encoding the RPI.As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (*e.g.*, for improved stability, targeting, etc.) and should be delivered to cells that express the RPI *in vivo*. A preferred method of delivery involves using a DNA construct encoding the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous mRNA encoding the RPI and inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is

required for efficiency. Endogenous RPI expression can also be reduced by inactivating or knockingout the RPI gene or its promoter using targeted homologous recombination (e.g., see Smithies, et al., 1985, Nature 317:230-234; Thomas and Capecchi, 1987, Cell 51:503-512; Thompson et al., 1989, Cell 5:313-321; each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional RPI gene (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous RPI gene (either the coding regions or regulatory regions of the gene encoding the RPI) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the target gene in vivo. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches are particularly suited in the agricultural field where modifications to ES (embryonic stem) cells can be used to generate animal offspring with an inactive target gene (e.g., see Thomas and Capecchi, 1987 and Thompson, 1989, supra,. However this approach can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site in vivo using appropriate viral vectors.Alternatively, endogenous RPI gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the RPI gene (i.e., the RPI gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the RPI gene in target cells in the body. (See generally, Helene, 1991, Anticancer Drug Des., 6(6), 569-584; Helene, et al., 1992, Ann. N.Y. Acad. Sci., 660, 27-36; and Maher, 1992, Bicassays 14(12), 807-815).Nucleic acid molecules to be used in triplex helix formation for the inhibition of transcription should be single stranded and

composed of deoxynuclectides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC' triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, contain a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called switchback nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.In instances wherein the antisense, ribozyme, and/or triple helix molecules described herein are utilized to inhibit mutant gene expression, it is possible that the technique may so efficiently reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal RPI gene alleles that the possibility may arise wherein the concentration of normal RPI gene product present may be lower than is necessary for a

normal phenotype. In such cases, to ensure that substantially normal levels of RPI gene activity are maintained, therefore, nucleic acid molecules that encode and express RPI gene polypeptides exhibiting normal RPI gene activity may, be introduced into cells via gene therapy methods that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively, in instances whereby the RPI gene encodes an extracellular protein, it may be preferable to co-administer normal RPI gene protein in order to maintain the requisite level of target gene activity.Anti-sense RNA and DNA, ribozyme, and triple helix molecules of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules, as discussed above. These include techniques for chemically synthesizing oligodecxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.Demonstration Of Therapeutic Or Prophylactic UtilityThe compounds of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays which can be used to determine whether administration of a specific compound is indicated, include *in vitro* cell culture assays

in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed. Test compounds can be tested for their ability to restore RADF or RPI levels in patients suffering from RA towards levels found in subjects not suffering from RA or to produce similar changes in experimental animal models of RA (e.g., adjuvant arthritis in rats). Compounds able to restore said levels can be used as lead compounds for further drug discovery, or used therapeutically. RADF and RPI expression can be assayed by the Preferred Technology, immunoassays, gel electrophoresis followed by visualization, or any other method known to those skilled in the art. Such assays can be used to screen candidate drugs or in clinical monitoring or drug development, where abundance of an RADF or RPI can serve as a surrogate marker for clinical disease.In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a compound has a desired effect upon such cell types.Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used. Therapeutic/Prophylactic

Administration And CompositionsThe invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a compound of the invention. In a preferred aspect, the compound is substantially purified (e.g. substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but

not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.Formulations and methods of administration that can be employed when the compound comprises a nucleic acid are described above; additional appropriate formulations and routes of administration can be selected from among those described hereinbelow.Various delivery systems are known and can be used to administer a compound of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, *J. Biol. Chem.* 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.In a specific embodiment, it may be desirable to

administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre-neoplastic tissue.In another embodiment, the compound can be delivered in a vesicle, in particular a liposome (*see* Langer, *Science* 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; *see* generally *ibid.*)In yet another embodiment, the compound can be delivered in a controlled release system. In one embodiment, a pump may be used (*see* Langer, *supra*; Sefton, *CRC Crit. Ref. Biomed. Eng.* 14:201 (1987); Buchwald et al., *Surgery* 88:507 (1980); Saudek et al., *N. Engl. J. Med.* 321:574 (1989)). In another embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, *J. Macromol. Sci. Rev. Macromol. Chem.* 23:61 (1983); *see also* Levy et al., *Science* 228:190 (1985); During et al., *Ann. Neurol.* 25:351 (1989); Howard et al., *J. Neurosurg.* 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target,

i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)). Other controlled release systems are discussed in the review by Langer (*Science* 249:1527-1533 (1990)). In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see *e.g.*, Joliot et al., 1991, *Proc. Natl. Acad. Sci. USA* 88:1864-1868), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination. The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term pharmaceuticallyacceptable means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term carrier refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin,

such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in Remington'sPharmaceuticalSciences by E.W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the

composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.The amount of the compound of the invention which will be effective in the treatment of RA can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal

administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. EXAMPLE PROTEINS FROM SERUM AND SYNOVIAL FLUID OF PATIENTS WITH RA Using the following Reference Protocol, proteins in serum and synovial fluid from patients with rheumatoid arthritis (RA) or from patients without RA (*i.e.*, patients with gout, osteoarthritis, or traumatic synovitis) were separated by isoelectric focusing followed by SDS-PAGE and compared. Each sample was run in duplicate.

Sample preparationA protein assay was carried out on the sample as received (Pierce BCA Cat # 23225). A volume of serum corresponding to 300µg of total protein was aliquoted and an equal volume of 10% (w/v) SDS (Fluka 71729), 2.3% (w/v) dithiothreitol (BDH 443852A) was added. The sample was heated at 95°C for 5 mins, and then allowed to cool to 20°C . 125µl of the following buffer was then added to the sample8M urea (BDH 452043w)4% CHAPS (Sigma C3023)65mM dithiotheitol (DTT)2% (v/v) Resolytes 3.5-10 (BDH 44338 2x)This mixture was vortexed, and centrifuged at 13000 rpm for 5 mins at 15°C, and the supernatant was analyzed by isoelectric

focusing. Isoelectric Focusing Isoelectric focusing (IEF), was performed using the Immobiline® DryStrip Kit (Pharmacia BioTech), following the procedure described in the manufacturer's instructions, see Instructions for Immobiline® DryStrip Kit, Pharmacia, # 18-1038-63, Edition AB (incorporated herein by reference in its entirety). Immobilized pH Gradient (IPG) strips (18cm, pH 3-10 non-linear strips; Pharmacia Cat. # 17-1235-01) were rehydrated overnight at 20°C in a solution of 8M urea, 2% (w/v) CHAPS, 10mM DTT, 2% (v/v) Resolytes 3.5-10, as described in the Immobiline DryStrip Users Manual. For IEF, 50µl of supernatant (prepared as above) was loaded onto a strip, with the cup-loading units being placed at the basic end of the strip. The loaded gels were then covered with mineral oil (Pharmacia 17-3335-01) and a voltage was immediately applied to the strips according to the following profile, using a Pharmacia EPS3500XL power supply (Cat 19-3500-01):

Initial voltage = 300V for 2 hrs
Linear Ramp from 300V to 3500V over 3hrs
Hold at 3500V for 19hrs

For all stages of the process, the current limit was set to 10mA for 12 gels, and the wattage limit to 5W. The temperature was held at 20°C throughout the run.

6.3 Gel Equilibration and SDS-PAGE Gel Equilibration and SDS-PAGE

1.1

After the final 19hr step, the strips were immediately removed and immersed for 10 mins at 20°C in a first solution of the following composition: 6M urea; 2% (w/v) DTT; 2% (w/v) SDS; 30% (v/v) glycerol (Fluka 49767); 0.05M Tris/HCl, pH 6.8 (Sigma Cat T-1503). The strips were removed from the first solution and immersed for 10 mins at 20°C in a second solution of the following composition: 6M urea; 2% (w/v) iodoacetamide (Sigma I-6125); 2% (w/v) SDS; 30% (v/v) glycerol; 0.05M Tris/HCl, pH 6.8. After removal from the second solution, the strips were loaded onto supported gels for SDS-PAGE according to Hochstrasser et al., 1988, *Analytical Biochemistry* 173:412-423 (incorporated herein by reference in its entirety), with modifications as specified below.

6.4 Preparation of supported gels Preparation of supported gels

The gels were cast between two glass plates of the following dimensions: 23cm wide x 24cm long (back plate); 23cm wide x 24cm long with a 2cm deep notch in the central 19cm (front plate). To promote covalent attachment of SDS-PAGE gels, the back plate was treated with a 0.4% solution of γ-methacryl-oxypropyltrimethoxysilane in ethanol (BindSilane™; Pharmacia Cat. # 17-1330-01). The front plate was treated with RepelSilane™ (Pharmacia Cat. # 17-1332-01) to reduce adhesion of the gel. Excess reagent was removed by washing with water, and the plates were allowed to dry. At this stage, both as identification for the gel, and as a marker to identify the coated face of the plate, an adhesive bar-code was attached to the back plate in a position such that it would not come into contact with the gel matrix.

The dried plates were assembled into a casting box with a capacity of 13 gel sandwiches. The top and bottom plates of each sandwich were spaced by means of 1mm thick spacers, 1.5 cm wide. The sandwiches were interleaved with acetate sheets to facilitate separation of the sandwiches after gel polymerization. Casting was then carried out according to Hochstrasser et al., op. cit.

A 9-16% linear polyacrylamide gradient was cast, extending up to a point 2cm below the level of the notch in the front plate, using the Angelique gradient casting system (Large Scale Biology). Stock solutions were as follows. Acrylamide (40% in water) was from Serva (Cat. # 10677). The cross-linking agent was PDA (BioRad 161-0202), at a concentration of 2.6% (w/w) of the total starting monomer content. The gel buffer was 0.375M Tris/HCl, pH 8.8. The polymerization catalyst was 0.05% (v/v) TEMED (BioRad 161-0801), and the initiator was 0.1% (w/v) APS (BioRad 161-0700). No SDS was included in the gel and no stacking gel was used. The cast gels were allowed to polymerize at 20°C overnight, and then stored at 4°C in sealed polyethylene bags with 6ml of gel buffer, and were used within 4 weeks.

6.5 SDS-PAGE SDS-PAGE

1.1

A solution of 0.5% (w/v) agarose (Fluka Cat 05075) was prepared in running buffer (0.025M Tris, 0.198M glycine (Fluka 50050), 1% (w/v) SDS, supplemented by a trace of bromophenol blue). The agarose suspension was heated to 70°C with stirring, until the agarose had dissolved. The top of the supported 2nd D gel was filled with the agarose solution, and the equilibrated strip was placed into the agarose, and tapped gently with a palette knife until the gel was

intimately in contact with the 2nd D gel. The gels were placed in the 2nd D running tank, as described by Amess et al., 1995, *Electrophoresis* 16:1255-1267 (incorporated herein by reference in its entirety). The tank was filled with running buffer (as above) until the level of the buffer was just higher than the top of the region of the 2nd D gels which contained polyacrylamide, so as to achieve efficient cooling of the active gel area. Running buffer was added to the top buffer compartments formed by the gels, and then voltage was applied immediately to the gels using a Consort E-833 power supply. For 1 hour, the gels were run at 20mA/gel. The wattage limit was set to 150W for a tank containing 6 gels, and the voltage limit was set to 600V. After 1 hour, the gels were then run at 40mA/gel, with the same voltage and wattage limits as before, until the bromophenol blue line was 0.5cm from the bottom of the gel. The temperature of the buffer was held at 10°C throughout the run.

6.6 Staining Staining

Upon completion of the electrophoresis run, the gels were immediately removed from the tank for fixation. The top plate of the gel cassette was carefully removed, leaving the gel bonded to the bottom plate. The bottom plate with its attached gel was then placed into a staining apparatus, which can accommodate 12 gels. The gels were completely immersed in fixative solution of 40% (v/v) ethanol (BDH 28719), 10% (v/v) acetic acid (BDH 100016X), 50% (v/v) water (MilliQ-Millipore), which was continuously circulated over the gels. After an overnight incubation, the fixative was drained from the tank, and the gels were primed by immersion in 7.5% (v/v) acetic acid, 0.05% (w/v) SDS, 92.5% (v/v) water for 30 mins. The priming solution was then drained, and the gels were

stained by complete immersion in a staining solution for 4 hours. A solution of fluorescent dye was prepared by diluting Sypro Red (Molecular Probes, Inc., Eugene, Oregon) according to the manufacturer's instructions; this diluted solution was filtered under vacuum though a 0.4um filter

6.7 Imaging of the gelcompletion of the electrophoresis run, the gels were immediately removed from the tank for fixation. The top plate of the gel cassette was carefully removed, leaving the gel bonded to the bottom plate. The bottom plate with its attached gel was then placed into a staining apparatus, which can accommodate 12 gels. The gels were completely immersed in fixative solution of 40% (v/v) ethanol (BDH 28719), 10% (v/v) acetic acid (BDH 100016X), 50% (v/v) water (MilliQ-Millipore), which was continuously circulated over the gels. After an overnight incubation, the fixative was drained from the tank, and the gels were primed by immersion in 7.5% (v/v) acetic acid, 0.05% (w/v) SDS, 92.5% (v/v) water for 30 mins. The priming solution was then drained, and the gels were stained by complete immersion in a staining solution for 4 hours. A solution of fluorescent dye was prepared by diluting Sypro Red (Molecular Probes, Inc., Eugene, Oregon) according to the manufacturer's instructions; this diluted solution was filtered under vacuum though a 0.4um filter. Imaging of the gel

1.1

A computer-readable output was produced by imaging the fluorescently stained gels with a Storm scanner (Molecular

Dynamics, Sunnyvale, California) according to the manufacturer's instructions, (*see* Storm User's Guide, 1995, Version 4.0, Part No. 149-355, incorporated herein by reference in its entirety) with modifications as described below. The gels were removed from the stain, rinsed with water briefly, and imaged on the Storm Scanner, in Red Fluorescence mode with a PMT setting of 1000V, and a resolution of 200 μm. Since the gel was rigidly bonded to a glass plate, the gel was held in contact with the scanner bed during imaging. To avoid interference patterns arising from non-uniform contact between the gel and the scanner bed, a film of water was introduced under the gel, taking care to avoid air pockets. Moreover, the gel was placed in a frame provided with two fluorescent buttons that were imaged together with the gel to provide reference points (designated M1 and M2) for determining the x,y coordinates of other features detected in the gel. A matched frame was provided on a robotic gel excisor in order to preserve accurate alignment of the gel. After imaging, the gels were sealed in polyethylene bags containing a small volume of staining solution, and then stored at 4°C.

6.8 Digital Analysis of the Data

The data were processed as described in U.S. Application No. 08/980,574, Sections 5.4 and 5.5 (incorporated herein by reference), as set forth more particularly below.

6.8.1 Computer Analysis Of The Detector Output Digital Analysis of the DataThe data were processed as described in U.S. Application No. 08/980,574, Sections 5.4 and 5.5 (incorporated herein by reference), as set

forth more particularly below. Computer Analysis Of The Detector Output

The output from the scanner was first processed using the MELANIE® II 2D PAGE analysis program (Release 2.2, 1997, BioRad Laboratories, Hercules, California, Cat. # 170-7566) to autodetect the registration points, M1 and M2; to autocrop the images (*i.e.*, to eliminate signals originating from areas of the scanned image lying outside the boundaries of the gel, *e.g.*, the reference frame); to filter out artifacts due to dust; to detect and quantify features; and to create image files in GIF format. Features were detected using the following parameters:

Smooths =2
Laplacian threshold 50
Partials threshold 1
Saturation = 100
Peakedness = 0
Minimum Perimeter = 10

6.9 Assignment of pI and MW Values Assignment of pI and MW Values

Images were evaluated to reject images which had gross abnormalities, or were of too low a loading or overall image intensity, or were of too poor a resolution, or where duplicates were too dissimilar. If one image of a duplicate was rejected then the other image belonging to the duplicate was also rejected regardless of image quality. Samples that were rejected were scheduled for repeat analysis.

Landmark identification was used to determine the pI and MW values of features detected in the images. This process involves the identification of certain proteins which are expected to be found in any given biological sample. As these common proteins exhibit an identical isoelectric point and molecular weight from sample to sample, they can be used

as standards; this process also corrects for any possible gel variation or distortion.

From the dataset of normal serum gels, a gel was arbitrarily chosen as the Primary Master Gel. Landmark features were then identified by comparing the features detected in this Primary Master Gel with features previously identified on 2D electrophoresis of normal human serum. (*see* Bjellqvist et al., 1993, *Electrophoresis* 14:1357-1365; incorporated herein by reference in its entirety).

Fourteen landmark features, designated PL1 to PL12 and PL15 to PL16, were identified in the Primary Master Gel. These landmark features are identified in Figure 1 and were assigned the pI and/or MW values indicated in Table XII.

Table XII. Landmark Features used in this study

Name	pI	MW (kd)	Name	pI	MW (kd)
PL1	None	186,073	PL8	6.47	47,195
PL2	6.20	100,000	PL9	5.29	43,541
PL3	4.73	93,708	PL10	5.22	23,000
PL4	5.13	73,465	PL11	4.47	25,183
PL5	4.97	52,739	PL12	5.52	13,800
PL6	4.10	None	PL15	7.80	36,962
PL7	4.80	40,997	PL16	8.58	None

As many of these landmarks as possible were identified in each gel image in the dataset.

All features in the Master gel were then assigned a pI value by linear interpolation/extrapolation (using the MELANIE II software) to the two nearest landmarks that had been assigned a pI value, and were assigned a MW value by

linear interpolation/extrapolation (using the MELANIE II software) to the MW of the two nearest landmarks that had been assigned a MW value. These features were also labelled with a unique number known as its Molecular Cluster Index (or MCI).

Secondary Master gels were chosen for both the RA serum and RA synovial gels. Features in these gels were paired with common features in the Master gel, using the algorithm supplied with the MELANIE II software, as described at Section A, pp. 8-10 of the MELANIE II 2D PAGE (Release 2.2) User Manual (The Melanie Group, Geneva, Switzerland). Features that have been paired are linked to the corresponding MCI, and hence to an associated pI and MW value. Unpaired features present in these secondary master gels were assigned pI and MW values by linear interpolation/extrapolation (using the MELANIE II software) with respect to the pI and MW of the landmarks. Additional unique entries were then created in the MCI for these features.

6.9.1 Construction of Profiles

Construction of Profiles

All gels in the dataset were now matched to the Primary and Secondary Master Gels, and paired features were linked to the corresponding entries in the Molecular Cluster Index.

Duplicate gels were then aligned via the landmarks and a matching process performed so as to pair identical spots on the duplicate gels. This provided increased assurance that subsequently measured isoelectric points and molecular weights were accurate, as paired spots demonstrated the reproducibility of the separation and also filtered out artefacts.

A measurement of the intensity of each protein spot was taken and stored. Each protein spot was assigned an identification code and matched to a spot on the Master gel.

The end result of this aspect of the analysis was the generation, for each duplicate set of gels representing a single serum or synovial fluid sample, of a digital profile which contained, for each identified spot: 1) a unique arbitrary identification code, 2) the x,y coordinates, 3) the isoelectric point, 4) the molecular weight, 5) the signal value, 6) the standard deviation for each of the preceding measurements, and 7) a pointer to the MCI of the spot on the master gel to which this spot was matched. By virtue of the Laboratory Information Management System (LIMS), this profile was traceable to the actual stored gel from which it was generated, so that proteins identified by computer analysis of gel profile databases could be retrieved. The LIMS also permitted the profile to be traced back to the original sample or patient.

6.9.2 Cross Matching Between Samples

1.1.1

Once the profile was generated, analysis was directed toward the selection of interesting proteins. Each significant feature in a profile was assigned an index, the "Molecular Cluster Index" (MCI) that identifies the feature in all gels and that serves as a pointer to parameters (1) to (7) above of the feature. A molecular cluster table was generated from the master gel for each sample type (e.g., RA serum and RA synovial fluid). Gels from all other samples of the same type were matched with the relevant primary and secondary master gels. The digital profiles for each sample

were then annotated by adding, for each matched feature, the MCI assigned to that feature in the master profile.

6.9.3 Differential Analysis of the Profiles

Within each sample set (synovial fluid or serum), the profiles were analyzed to identify and select those features present in at least 50% of the profiles. These selected features were then assembled into a synovial fluid feature set and a serum feature set. Matching features of each feature set were then compared to identify those features showing at least a 2-fold difference in mean intensity between synovial fluid and serum. Then, the same features were examined in the synovial and serum samples from subjects without RA. Features which were differentially present in the RA serum as compared to the RA synovial fluid samples but not differentially present in the non-RA serum as compared to the non-RA synovial fluid samples were identified as Rheumatoid Arthritis-Diagnostic Features (RADFs).

6.10 Recovery and analysis of selected proteinsRecovery and analysis of selected proteins

Proteins in RADFs were robotically excised and processed to generate tryptic peptides; partial amino acid sequences of these peptides were determined by mass spectroscopy, using de novo sequencing.

6.11 Results Results

1.1

These initial experiments identified 12 features that were increased and 10 features that were decreased in synovial fluid verses serum from RA patients. Each RADF was

differentially present only in synovial fluid verses serum from subjects with RA but not in synovial fluid verses serum from subjects without RA.

Partial amino acid sequences were determined for the differentially present RPIs in these RADFs. Computer analysis of public databases revealed that 21 of these partially sequenced proteins were known in the art and that 5 were not described in any public database examined. Table VIII illustrates that several RPIs are isoforms of the same protein. For example, RPI-1 and RPI-11 are isoforms of transferrin. These isoforms are thought to arise from differences in post-translational processing (*e.g.*, glycosylation, phosphorylation, acylation or minimal proteolysis).

7 EXAMPLE: PROTEINS FROM SERUM OF PATIENTS WITH AND WITHOUT RA

Proteins in serum from patients with rheumatoid arthritis (RA) and from patients without RA were separated by isoelectric focusing followed by SDS-PAGE and compared.

The analysis was performed as described in Example 6, except that the comparison in Example 7 was between serum from RA patients and serum from non-RA patients.

7.1 Results

These initial experiments identified 12 features that were increased and 9 features that were decreased in serum from RA patients as compared with serum from non-RA patients. Details of these RADFs are provided in Tables III and IV. Each RADF was differentially present in RA serum versus non-RA serum.

Partial amino acid sequences were determined for the differentially present RPIs in these RADFs. Computer

analysis of public databases revealed that 17 of these partially sequenced proteins were known in the art and that 4 were not described in any public database examined. Table XI illustrates that several RPIs are isoforms of the same protein. For example, RPI-44 and RPI-45 are isoforms of transferrin. These isoforms are thought to arise from differences in post-translational processing (e.g., glycosylation, phosphorylation, acylation or minimal proteolysis).

Moreover, Tables VIII to XI demonstrate that RPI-2, RPI-6, RPI-7, RPI-14, RPI-23, RPI-25, RPI-31, and RPI-37 represent immunoglobulin isoforms. RPI-2, in particular represents an IgG light chain isoform. In other words, the Preferred Technology has been used to identify a defined subset of immunoglobulin isoforms that are specifically associated with RA and that likely reflect an oligoclonal humoral immune response associated with this disease. These immunoglobulin isoforms (and fragments thereof, antibodies thereto, etc.) are useful for diagnosis, prognosis, therapeutic monitoring and drug development.

The present invention is not to be limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All publications cited herein are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method for screening, diagnosis or prognosis of RA in a subject or for monitoring the effect of an anti-RA drug or therapy administered to a subject, comprising:

(a) analyzing a sample of serum or plasma from the subject by two-dimensional electrophoresis to generate a two-dimensional array of features;

(b) for at least one chosen feature whose relative abundance correlates with the presence or absence of RA, comparing the abundance of each chosen feature in the sample with the abundance of that chosen feature in serum or plasma from one or more persons without RA,

wherein the relative abundance of the chosen feature or features in the sample indicates the presence or absence of RA in the subject.

2. A method for screening, diagnosis or prognosis of RA in a subject or for monitoring the effect of an anti-RA drug or therapy administered to a subject, comprising:

(a) analyzing a sample of synovial fluid from the subject by two-dimensional electrophoresis to generate a two-dimensional array of features;

(b) analyzing a sample of serum or plasma from the subject by two-dimensional electrophoresis to generate a two-dimensional array of features;

(c) for at least one chosen feature whose relative abundance correlates with the presence or absence of RA, comparing the abundance of each chosen feature in the sample of synovial fluid with the abundance of that chosen feature in the sample of serum or plasma,

wherein the relative abundance of the chosen feature or features in the synovial fluid sample as compared with the serum or plasma sample indicates the presence or absence of RA in the subject.

3. A method for screening, diagnosis or prognosis of RA in a subject or for monitoring the effect of an anti-RA drug or therapy administered to a subject, comprising:

(a) analyzing a sample of synovial fluid, serum or plasma from the subject by two-dimensional electrophoresis to generate a two-dimensional array of features;

(b) for at least one chosen feature whose intensity correlates with the presence or absence of RA, comparing the intensity of each chosen feature in the sample with the intensity of one or more chosen expression reference features (ERFs) in synovial fluid, serum or plasma from the subject,

wherein the intensity of the chosen feature or features in the sample relative to said one or more ERFs indicates the presence or absence of RA in the subject.

4. A method for screening, diagnosis or prognosis of RA in a subject or for monitoring the effect of an anti-RA drug or therapy administered to a subject, comprising:

(a) analyzing a sample of synovial fluid, serum or plasma from the subject by two-dimensional electrophoresis to separate a plurality of proteins according to isoelectric point and electrophoretic mobility; and

(b) quantitatively detecting at least one of the following RA-Diagnostic Features (RADFs): RADF-1, RADF-2, RADF-3, RADF-4, RADF-5, RADF-6, RADF-7, RADF-8, RADF-9, RADF-10, RADF-11, RADF-12, RADF-13, RADF-14, RADF-15, RADF-16, RADF-17, RADF-18, RADF-19, RADF-20, RADF-21, RADF-22, RADF-

23, RADF-24, RADF-25, RADF-26, RADF-27, RADF-28, RADF-29, RADF-30, RADF-31, RADF-32, RADF-33, RADF-34, RADF-35, RADF-36, RADF-37, RADF-38, RADF-39 and RADF-40.

5. The method according to claims 1, 2, 3 or 4, wherein step (a) comprises isoelectric focussing followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

6. A method for screening, diagnosis or prognosis of RA in a subject or for monitoring the effect of an anti-RA drug or therapy administered to a subject, comprising:

(a) in a sample of synovial fluid, serum or plasma from the subject, quantitatively detecting at least one of the following RA-Diagnostic Protein Isoforms (RPIs): RPI-1, RPI-2, RPI-3, RPI-4, RPI-5, RPI-6, RPI-8, RPI-9, RPI-10, RPI-11, RPI-12, RPI-13, RPI-14, RPI-15, RPI-16, RPI-17, RPI-18, RPI-19, RPI-20, RPI-21 or RPI-22, RPI-23, RPI-24, RPI-25, RPI-26, RPI-27, RPI-28, RPI-29, RPI-30, RPI-31, RPI-32, RPI-33, RPI-34, RPI-35, RPI-36, RPI-37, and RPI-38.

7. The method according to claim 6, wherein the step of quantitatively detecting comprises testing at least one aliquot of the sample, said step of testing comprising:

(a) contacting the aliquot with an antibody that is immunospecific for a preselected RPI; and

(b) detecting whether binding has occurred between the antibody and at least one species in the aliquot.

8. The method according to claim 7, wherein the antibody is a monoclonal antibody.

9. The method according to claim 7, wherein the step of quantitatively detecting comprises testing a plurality of aliquots with a plurality of antibodies.

10. The method according to claim 9, wherein the antibodies are monoclonal antibodies.

11. A preparation comprising one of the following isolated RA-Diagnostic Protein Isoforms (RPIs): RPI-1, RPI-2, RPI-3, RPI-4, RPI-5, RPI-6, RPI-8, RPI-9, RPI-10, RPI-11, RPI-12, RPI-13, RPI-14, RPI-15, RPI-16, RPI-17, RPI-18, RPI-19, RPI-20, RPI-21 or RPI-22, RPI-23, RPI-24, RPI-25, RPI-26, RPI-27, RPI-28, RPI-29, RPI-30, RPI-31, RPI-32, RPI-33, RPI-34, RPI-35, RPI-36, RPI-37, or RPI-38.

12. A kit comprising the preparation of claim 11.

14. A kit comprising a plurality of preparations of claim 11.

15. A preparation comprising an isolated human protein, said protein comprising a peptide having one of the following sequences: VAAIEHFGR or VAALEHFGR.

16. The preparation according to claim 15, wherein the protein has an isoelectric point (pI) of about 5.98 and an apparent molecular weight (MW) of about 52,631.

17. The preparation according to claim 16, wherein the pI is within 10% of 5.98 and the MW is within 10% of 52,631.

18. The preparation according to claim 17, wherein the pI is within 5% of 5.98 and the MW is within 5% of 52,631.

19. The preparation according to claim 18, wherein the pI is within 1% of 5.98 and the MW is within 1% of 52,631.

20. A preparation comprising an isolated human protein, said protein comprising a peptide having one of the following sequences: DSGADIS or DSGADLS.

21. The preparation according to claim 20, wherein the protein has an isoelectric point (pI) of about 5.36 and an apparent molecular weight (MW) of about 24,124.

22. The preparation according to claim 21, wherein the pI is within 10% of 5.36 and the MW is within 10% of 24,124.

23. The preparation according to claim 22, wherein the pI is within 5% of 5.36 and the MW is within 5% of 24,124.

24. The preparation according to claim 23, wherein the pI is within 1% of 5.36 and the MW is within 1% of 24,124.

25. A preparation comprising an isolated human protein, said protein comprising a peptide having one of the following sequences: NVIDAPHAR or NVLDAPHAR.

26. The preparation according to claim 25, wherein the protein has an isoelectric point (pI) of about 5.96 and an apparent molecular weight (MW) of about 158,868.

27. The preparation according to claim 26, wherein the pI is within 10% of 5.96 and the MW is within 10% of 158,868.

28. The preparation according to claim 27, wherein the pI is within 5% of 5.96 and the MW is within 5% of 158,868.

29. The preparation according to claim 28, wherein the pI is within 1% of 5.96 and the MW is within 1% of 158,868.

30. An antibody capable of immunospecific binding to one of the following RA-Diagnostic Protein Isoforms (RPIs): RPI-1, RPI-2, RPI-3, RPI-4, RPI-5, RPI-6, RPI-8, RPI-9, RPI-10, RPI-11, RPI-12, RPI-13, RPI-14, RPI-15, RPI-16, RPI-17, RPI-18, RPI-19, RPI-20, RPI-21 or RPI-22, RPI-23, RPI-24, RPI-25, RPI-26, RPI-27, RPI-28, RPI-29, RPI-30, RPI-31, RPI-32, RPI-33, RPI-34, RPI-35, RPI-36, RPI-37, and RPI-38.

31. A kit comprising the antibody of claim 30.

32. A kit comprising a plurality of antibodies of claim 30.

33. A pharmaceutical composition comprising a therapeutically effective amount of one or more of the following isolated RA-Diagnostic Protein Isoforms (RPIs): RPI-12, RPI-13, RPI-14, RPI-15, RPI-16, RPI-17, RPI-18, RPI-19, RPI-20, RPI-21, RPI-22, RPI-23, and RPI-24.

34. A pharmaceutical composition comprising a therapeutically effective amount of an antibody that immunospecifically binds to one of the following RA-Diagnostic Protein Isoforms (RPIs): RPI-1, RPI-2, RPI-3, RPI-4, RPI-5, RPI-6, RPI-8, RPI-9, RPI-10, or RPI-11.

35. A pharmaceutical composition comprising a therapeutically effective amount of a fragment or derivative

of an antibody that immunospecifically binds to one of the following RA-Diagnostic Protein Isoforms (RPIs): RPI-1, RPI-2, RPI-3, RPI-4, RPI-5, RPI-6, RPI-8, RPI-9, RPI-10, or RPI-11, said fragment or derivative containing the binding domain of the antibody; and a pharmaceutically acceptable carrier.

36. A method of treating or preventing RA comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of a nucleic acid encoding one of the following RA-Diagnostic Protein Isoforms (RPIs): RPI 12, RPI-13, RPI-14, RPI-15, RPI-16, RPI-18, RPI-19, RPI-21, RPI-22, RPI-23, or RPI-24.

37. A method of treating or preventing RA comprising administering to a subject in need of such treatment or prevention a therapeutically effective amount of a nucleic acid that inhibits the function of one or more of the following RA-Diagnostic Protein Isoforms (RPIs): RPI-1, RPI-2, RPI-3, RPI-4, RPI-5, RPI-6, RPI-8, RPI-9, or RPI-11.

38. The method of claim 37, wherein the nucleic acid is an RPI anti-sense nucleic acid or ribozyme.

39. The use of one or more RADFs, as defined in claim 4 in screening, diagnosis or prognosis of RA in a subject or for monitoring the effect of an anti-RA drug or therapy administered to a subject.

40. The use of one or more RPIs, as defined in claim 6, in screening, diagnosis or prognosis of RA in a subject or for monitoring the effect of an anti-RA drug or therapy

administered to a subject.

41. The use of at least one antibody immunospecific for an RPI, as defined in claim 6, in screening, diagnosis or prognosis of RA in a subject or for monitoring the effect of an anti-RA drug or therapy administered to a subject.

42. The use as claimed in claim 41 wherein the at least one antibody is a monoclonal antibody.

43. The use of a protein as defined in any one of claims 15 to 29 in screening, diagnosis or prognosis of RA in a subject or for monitoring the effect of an anti-RA drug or therapy administered to a subject.

44. The use of at least one RPI as defined in claim 33 in the preparation of a medicament for use in the prevention or treatment of RA.

45. The use of at least one antibody as defined in claim 34 in the preparation of a medicament for use in the prevention or treatment of RA.

46. The use of a fragment or derivative of an antibody as defined in claim 35 in the preparation of a medicament for use in the prevention or treatment of RA.

47. The use of a nucleic acid as defined in claim 36 in the preparation of a medicament for use in the prevention or treatment of RA.

48. The use of a nucleic acid as defined in claim 37 in the

preparation of a medicament for use in the prevention or treatment of RA.

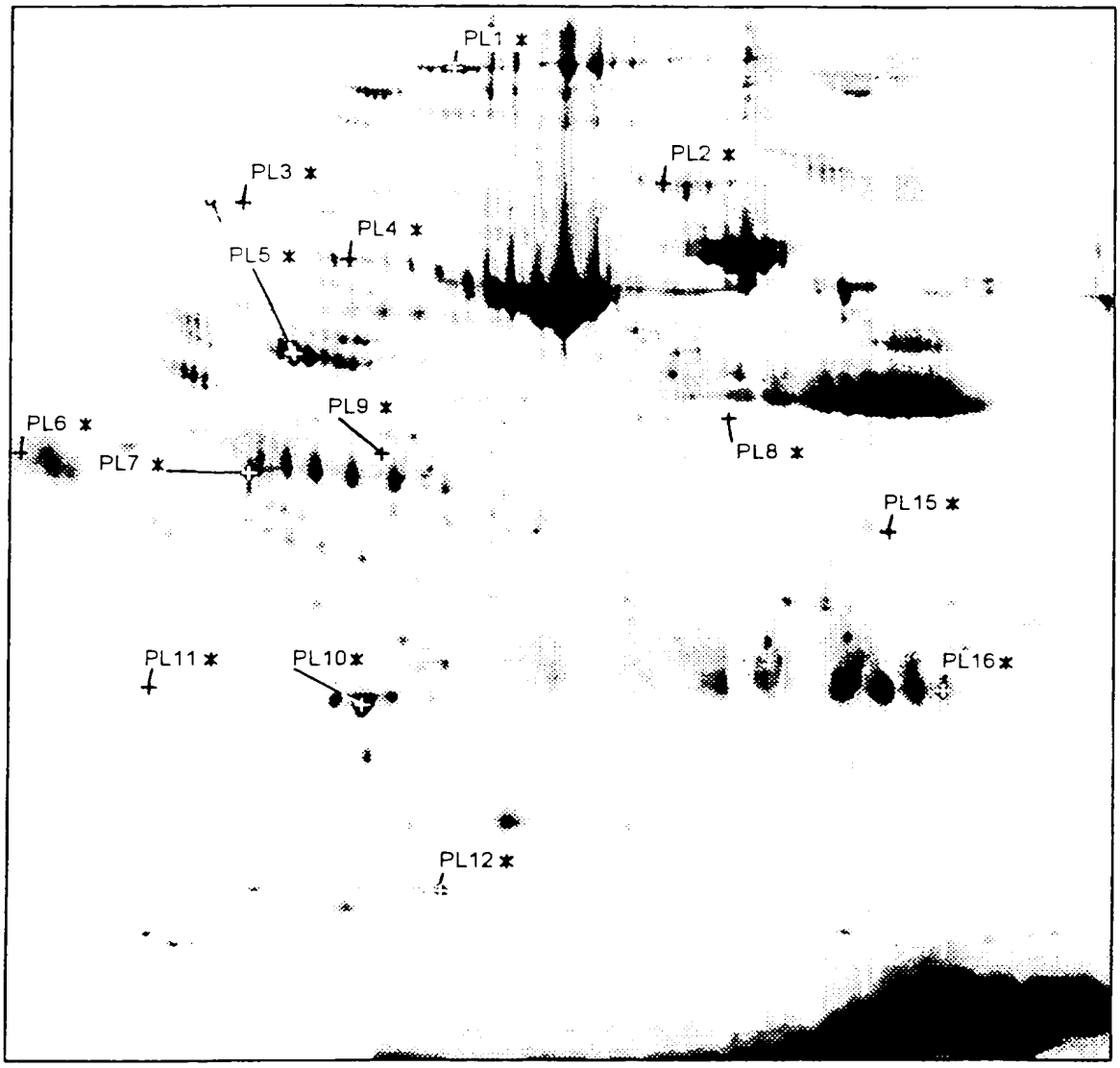

FIG. 1

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(54) Title: METHODS AND COMPOSITIONS FOR DIAGNOSIS OF RHEUMATOID ARTHRITIS

(57) Abstract

The present invention provides methods and compositions for screening, diagnosis and prognosis of RA, for monitoring the effectiveness of RA treatment, and for drug development. RA–Diagnostic Features (RADFs), detectable by two–dimensional electrophoresis of serum or plasma are described. The invention further provides RA–Diagnostic Protein Isoforms (RPIs) detectable in synovial fluid, serum or plasma, preparations comprising isolated RPIs, antibodies immunospecific for RPIs, and kits comprising the aforesaid.

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INTERNATIONAL SEARCH REPORT

International Application No
PC1/GB 99/00763

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/564 C07K14/47 A61K38/17 C07K16/18 A61K39/395
A61K31/70 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 378 732 A (ACRAF) 25 July 1990 (1990-07-25) column 2, line 24-41; claims 4,12	1,4-12, 30,31, 34,35, 37,39-48
Y	the whole document column 4, line 16 - line 29 ---	1-48
Y	WO 95 16919 A (MATRITECH INC) 22 June 1995 (1995-06-22) the whole document ---	1-48
Y	WO 98 09170 A (MATRITECH INC) 5 March 1998 (1998-03-05) claim 1; figures 1,2; example 1 --- -/--	1-48

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 September 1999	13/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hoekstra, S

3

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00763

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 175 310 A (ASAHI MEDICAL CO ;MEDECS CO LTD (JP)) 26 March 1986 (1986-03-26) figures 1,2	1,5
X	EKI, TOSHIHIKO ET AL: "Fifteen open reading frames in a 30.8 kb region of the right arm of chromosome VI from Saccharomyces cerevisiae" YEAST (1996), 12(2), 177-90 , XP002116970 the whole document	20-24
X	MURAKAMI, YASUFUMI ET AL: "Analysis of the nucleotide sequence of chromosome VI from Saccharomyces cerevisiae" NAT. GENET. (1995), 10(3), 261-8 , XP002116971 the whole document	20-24
X	HOSSEINI-MAZINANI, S. M. ET AL: "Cloning and sequencing of sulfite reductase.alpha.-subunit gene from Saccharomyces cerevisiae" DNA RES. (1995), 2(1), 15-19 , XP002116972 the whole document	20-24
X	HANSEN, JORGEN ET AL: "Two divergent MET10 genes, one from Saccharomyces cerevisiae and one from Saccharomyces carlsbergensis, encode the.alpha. subunit of sulfite reductase and specify potential binding sites for FAD and NADPH" J. BACTERIOL. (1994), 176(19), 6050-8 , XP002116973 the whole document	20-24
P,A	US 5 837 686 A (STANWORTH DENIS R ET AL) 17 November 1998 (1998-11-17) column 1 -column 2	1-48

3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 99/00763

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see Further information

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see Further information

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see Further information

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-10, 39-48

   Methods for screening, diagnosis, or prognosis of rheumatoid arthritis or monitoring the effect of an anti-rheumatoid arthritis drug of therapy involving 2-D electrophoresis of a body fluid and comparing levels of individual spots identified in the 2-D gel with samples from othe body fluids, e.g. form a body fluid from a person without rheumatoid arthritis.

2. Claims: 11-14, 30-32, 34, 35, 43 (partly), 15-19 entirely

   Preparations and compositions characterised by the presence of a protein comprising the peptide VAA(I/L)EHFGR (RPI-10;RADF-11) only.

3. Claims: 11-14, 30-33, 35, 43 (partly), 20-24 entirely

   Preparations and compositions characterised by the presence of a protein comprising the peptide DSGAD(I/L)S (RPI-17;RADF-16) only.

4. Claims: 11-14, 30-33, 35, 43 (partly), 25-29 entirely

   Preparations and compositions characterised by the presence of a protein comprising the peptide NV(I/L)DAPHAR (RPI-20;RADF-17)only.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 36-38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the nucleic acids insofar they realte to subejct-matter not excluded under Article 17(2)(a)(ii) PCT.

Continuation of Box I.1

Claims Nos.: 36-38

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

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Continuation of Box I.2

Claims Nos.: 4-10, 39-48, 11-14, 30-38 (All partly)

The subject-matter of claims 4 is characterised by arbitrary designations. This is contrary to the requirements of Article 6 and Rule 6.3(a) PCT. Due to the failure to comply with this prescribed requirement a meaningful search over the whole claimed range is not possible.

Moreover, the description fails to identify at least one RPI or combinations of RPI's as a solution to the technical problem of providing rheumatoid arthritis markers, therapeutic molecules or antigens. The description merely provides a choice of possibly relevant proteins, most of them already known per se, as is inferred from Tables VIII, IX, X and XI. As no technical indication is given as to which element or specified combination of elements, actually contributes to a solution (as required by Rule 5.1(iii) and Article 5 PCT) to a problem identifiable in the description a meaningful search is not possible.

The subject-matter of claims directed to preparations and compositions per se are characterised by partial sequences only and the vast majority of the "isoforms" comprise known preparations (Tables VIII, IX, X and XI). These claims (11-14, 30-38) can hence not be considered as defining the matter for which protection is sought which is nevertheless a major requirement laid down in Article 6 PCT, first sentence. A search for the subject-matter of these claims can not be meaningful.

The search has been limited to those claims, or part of claims, that define the matter for which protection can legitimately be sought as a solution to the problem of providing further methods for screening, diagnosis, or prognosis of rheumatoid arthritis or monitoring the effect of an anti-rheumatoid arthritis drug or therapy involving 2-D electrophoresis of a body fluid and comparing levels of individual spots identified in the 2-D gel with samples from other body fluids.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Also searched are the three highlighted RPI's of claims 15-29 which distinguish themselves from the others by the alleged novelty and which are thus the only ones which could legitimately lead to novel solutions to the technical problem the application is set out to solve.

With regard to Non-Unity the following may be noted:

The only feature shared by all claimed inventions is the involvement of at least one protein detected via 2-D gelelectrophoresis in a body fluid of a rheumatoid arthritis patient which protein is differentially expressed between patients and non-patients or between different body fluids of a candidate patient.
The single general concept covering all claimed inventions relates to the problem of detection and provision of a marker for rheumatoid arthritis.
The solution provided by the first invention is the use of 2-D gelelectrophoresis to identify differentially expressed or modified proteins between patients and non-patients.
The solutions of claim 15, 20 and 25 are preparations comprising at least one specified protein.

The single general concept identified above is not novel in view of EP378732 (col. 2, lines 24-41) and can hence not be the single general concept mentioned in Rule 13.1 PCT.
EP378732 discloses the identification, by way of 2-D gelelectrophoresis, of alphal-antitrypsin (identified in the present applications as RPI-3 or RADF-3) as a marker for rheumatoid arthritis.

Moreover, the first invention involves method features as special technical features distinguishing the claimed method from the method per se of EP378732 whereas each of the second third and fourth inventions comprise as special technical features the specific proteins per se as alternative solutions to the underlying technical problem of providing markers for rheumatoid arthritis other than alphal-antitrypsin. These special technical feautres are not shared by any pair of the four inventions (Rule 13.2 PCT).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 99/00763

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0378732 A	25-07-1990	EP 0585960 A	09-03-1994
		GR 90300168 T	27-09-1991
		US 5047509 A	10-09-1991
		US 5284750 A	08-02-1994
		US 5258312 A	02-11-1993
WO 9516919 A	22-06-1995	US 5547928 A	20-08-1996
		AU 1437895 A	03-07-1995
		US 5830677 A	03-11-1998
WO 9809170 A	05-03-1998	US 5858683 A	12-01-1999
		AU 4073297 A	19-03-1998
		EP 0923740 A	23-06-1999
EP 0175310 A	26-03-1986	JP 62016499 A	24-01-1987
		JP 1869293 C	06-09-1994
		JP 5082399 B	18-11-1993
		JP 61070463 A	11-04-1986
		JP 61070464 A	11-04-1986
		US 4950741 A	21-08-1990
		US 4863850 A	05-09-1989
		US 4742157 A	03-05-1988
US 5837686 A	17-11-1998	AT 163939 T	15-03-1998
		AU 2951392 A	28-06-1993
		AU 7196596 A	06-02-1997
		CA 2123694 A	10-06-1993
		DE 69224750 D	16-04-1998
		EP 0614464 A	14-09-1994
		ES 2112921 T	16-04-1998
		FI 942392 A	24-05-1994
		WO 9311153 A	10-06-1993
		GB 2261665 A,B	26-05-1993
		JP 7507045 T	03-08-1995
		AU 7097494 A	20-12-1994
		WO 9427643 A	08-12-1994
		US 5736387 A	07-04-1998